assistance of Mrs. Audrey Eisenstadt and Miss Elizabeth Schroeder during the course of this investigation.

References

Adams, J. E., and Capecchi, M. R. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 147.

Barondes, S. H., and Nirenberg, M. W. (1962), *Science 138*, 810.

Brawerman, G. (1963), *Biochim. Biophys. Acta 72*, 317.

Brawerman, G., Biezunski, N., and Eisenstadt, J. M. (1965), *Biochim. Biophys. Acta 103*, 201.

Eisenstadt, J., and Brawerman, G. (1964), *Biochim. Biophys. Acta 80*, 463.

Eisenstadt, J., and Brawerman, G. (1966), *Biochemistry 5*, 2777 (this issue; preceding paper).

Haselkorn, R., and Fried, V. A. (1963), *Proc. Natl. Acad. Sci. U. S. 51*, 308, 1001.

Marcker, K. (1965), *J. Mol. Biol. 14*, 63.

Marcker, K., and Sanger, F. (1964), *J. Mol. Biol. 8*, 835.

Schwartz, J. H., Eisenstadt, J. M., Brawerman, G., and Zinder, N. D. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 195.

Webster, R. E., Engelhardt, D. L., and Zinder, N. D. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 155.

The Effect of Sodium Chloride on Esterification of Leucine to Transfer Ribonucleic Acid by Heterologous Aminoacyl Transfer Ribonucleic Acid Synthetases*

Alan Peterkofsky, Suzanne J. Gee, and Celia Jesensky

ABSTRACT: The esterification of leucine to *Escherichia coli* or yeast transfer ribonucleic acid (t-RNA) by their respective homologous enzymes is unaffected by NaCl. However, the heterologous esterification reaction is markedly influenced by NaCl. In the presence of 0.12 M NaCl, both the rate of esterification and the yield of leucyl-t-RNA are appreciably inhibited. The level of inhibition is related to the concentration of NaCl. The evidence suggests that the leucyl-t-RNA synthetase undergoes a reversible modification in the presence of NaCl to a form that can no longer attach leucine to heterologous t-RNA. The pyrophosphate exchange reaction of the yeast leucyl-t-RNA synthetase is unaffected by NaCl. The K_m for *E. coli* leucine acceptor RNA is approximately 2.5×10^{-8} M for the *E. coli* enzyme and 1.6×10^{-7} M for the yeast enzyme. The K_m for yeast leucine acceptor RNA for the yeast enzyme is 4×10^{-7} M.

The initial enzymatic step unique to the process of protein synthesis involves the esterification of the free amino acid to t-RNA[1] (Hoagland *et al.*, 1958). While there is considerable evidence that there is a single aminoacyl-t-RNA synthetase for each of the naturally occurring amino acids (Berg, 1961),[2] there appear to be multiple species of t-RNA for the individual amino acids. It is assumed that the various species of t-RNA which can carry a single amino acid have the capacity to find their way to different positions in a protein sequence as a result of a unique interaction with template RNA. The process by which an aminoacyl-t-RNA synthetase selects the proper species of t-RNA for acylation presents an interesting problem in protein–nucleic acid interaction. Although a number of investigators have purified aminoacyl-t-RNA synthetases[3] and purified species of t-RNA are becoming increasingly available (Brown, 1963; Goldstein *et al.*, 1964; Muench and Berg, 1966), a clear picture of the mechanisms of t-RNA "recognition" or "discrimination" has not been formulated. A supplementary approach to the study of recognition of t-RNA by aminoacyl-t-RNA synthetases involves the use of heterologous enzymes (Yamane and Sueoka, 1963; Peterkofsky, 1964). The present work describes the effect of NaCl in specifically inhibiting heterologous aminoacylation of t-RNA and describes some experiments designed to characterize this phenomenon.

* From the Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014. *Received May 2, 1966.*

[1] Abbreviations used in this work: ATP, adenosine triphosphate; GSH, glutathione; PP_i, inorganic pyrophosphate; t-RNA, amino acid acceptor RNA; leucyl-t-RNA, the esterified form of the t-RNA; TCA, trichloroacetic acid.

[2] Yu and Rappaport (1966) have recently presented evidence for two distinct leucyl-t-RNA synthetases in *E. coli*.

[3] The recent paper by Baldwin and Berg (1966) includes a summary of references to these enzymes.

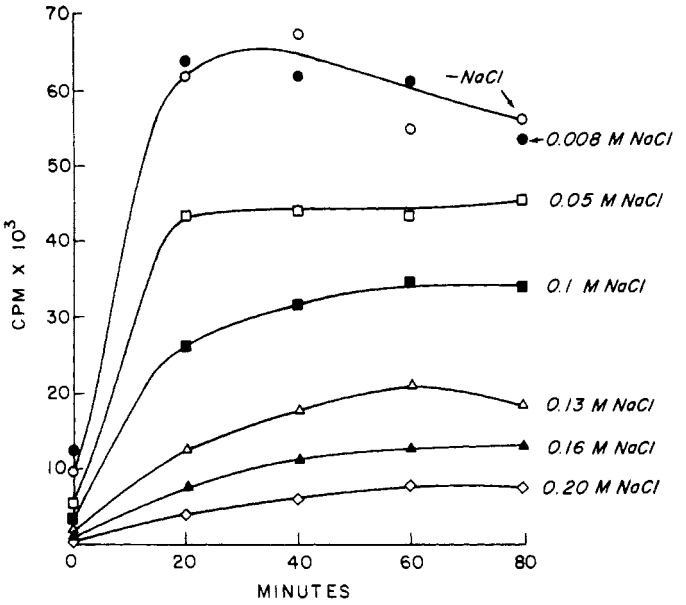

FIGURE 1: Effect of NaCl concentration on leucine acylation to *E. coli* t-RNA by yeast leucyl-t-RNA synthetase. Incubation mixtures were prepared in a total volume of 1.2 ml and contained, in micromoles: potassium cacodylate, pH 7.0, 30; ATP, 12; $MgCl_2$, 20; GSH, 21; [^{14}C]leucine, 0.004; and yeast enzyme, 0.04 ml. Where indicated, NaCl in the specified concentrations was included in the incubations. After preincubating at 37° for 45 min, the acylation reaction was initiated by the addition of 30 A_{260} of *E. coli* t-RNA and incubation was continued at 37°. At the indicated times, 0.2-ml aliquots were withdrawn, treated with TCA, and counted as in Table I.

Materials

Escherichia coli B was purchased as a frozen paste from Grain Processing Co., Muscatine, Iowa, and stored at −20°. Leucyl-t-RNA synthetase was prepared from these cells by a previously described procedure (Lazzarini and Peterkofsky, 1965) to yield a preparation containing approximately 10 mg/ml of protein. A strain of bakers yeast was generously provided by Dr. N. Sueoka, Department of Biology, Princeton University. Growth of the organism and preparation of the enzyme extract free from RNA were as described by Yamane and Sueoka (1963). The extract was dialyzed against 0.005 M phosphate buffer, pH 7.0, to give a preparation with approximately 3 mg/ml of protein. Both enzyme preparations were stored in small aliquots under liquid nitrogen. Soluble ribonucleic acid (stripped of amino acids) from *E. coli* B or yeast was obtained from General Biochemicals, Chagrin Falls, Ohio. In order to decrease contamination with nuclease, the commercial RNA preparations were further treated with phenol, reprecipitated with 20% potassium acetate (0.1 volume) and ethanol (2 volumes), then dialyzed overnight against water. GSH was purchased from Schwarz Bio-Research, Inc., as the sodium salt. The sodium salt of ATP was a product of Sigma Chemical Co., and 2-mercaptoethanol was from Eastman Organic Chemicals. Radioactive amino acids and $^{32}PP_i$ were purchased from New England Nuclear Corp.

TABLE I: Variability of Level of Leucine Esterification to *E. coli* t-RNA by Yeast Leucyl-t-RNA Synthetase.

Conditions	Cpm Incorp/A_{260}
2-Mercaptoethanol system[a]	6,850
GSH system[b]	11,900
GSH system + 0.12 M NaCl	7,725

[a] The 2-mercaptoethanol incubation system (0.2 ml) contained, in micromoles: potassium cacodylate, pH 7.0, 20; ATP (sodium salt), 2; $MgCl_2$, 8; 2-mercaptoethanol, 0.2; [^{14}C]leucine (220 mc/mmole), 0.002; *E. coli* t-RNA, 9.2 A_{260}; yeast enzyme, 0.05 ml. [b] The GSH incubation system (0.4 ml) contained, in micromoles: potassium cacodylate, pH 7.0, 10; ATP, 4; $MgCl_2$, 7; GSH (sodium salt), 7; [^{14}C]leucine, 0.002; *E. coli* t-RNA, 9.2 A_{260}; yeast enzyme, 0.05 ml. Incubation was at 37°. At various time intervals, reactions were terminated by the addition of 1 ml of 10% TCA. The precipitated aminoacyl-t-RNA was collected on Millipore filters and counted as previously described (Peterkofsky *et al.*, 1964). The results shown represent the maximum incorporation.

Methods

Protein was determined by the method of Lowry

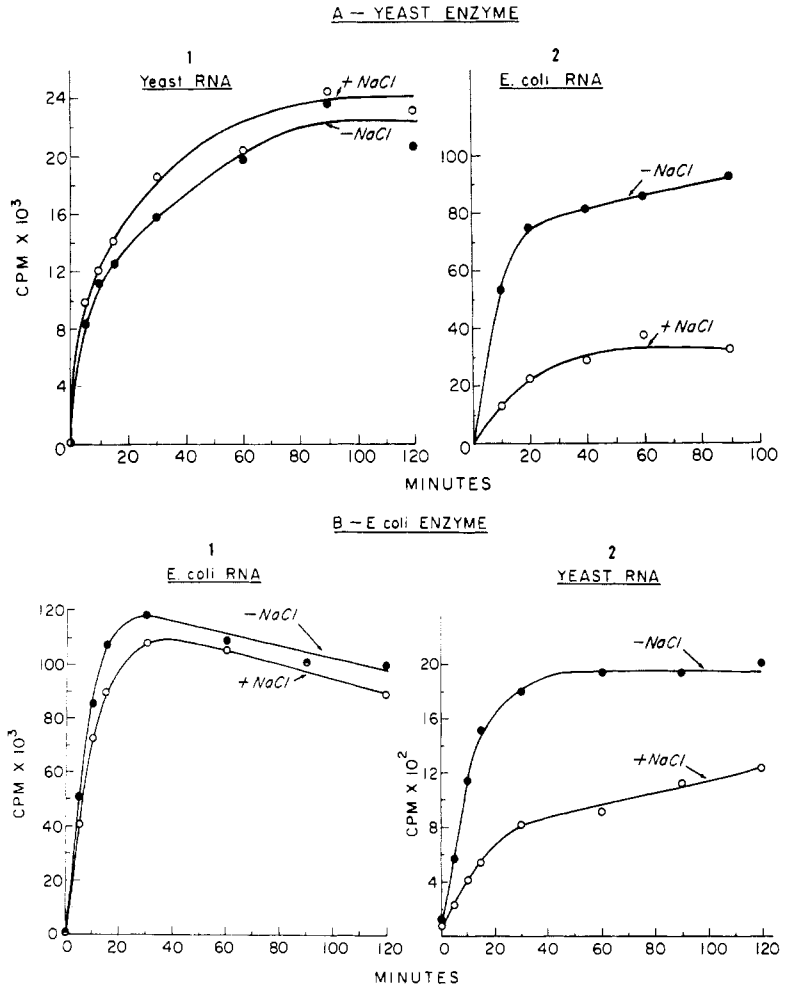

FIGURE 2: Effect of NaCl on leucine acylation to yeast or *E. coli* t-RNA by yeast or *E. coli* leucyl-t-RNA synthetase. Incubation mixtures were prepared in a total volume of 3.6 ml and contained in micromoles: potassium cacodylate, 90; ATP, 36; $MgCl_2$, 63; GSH, 63; [^{14}C]leucine, 0.018; enzyme, RNA and where indicated, 0.12 M NaCl. In A, the amount of yeast enzyme was 0.08 ml. In B, the amount of *E. coli* enzyme was 0.01 ml; where added, yeast t-RNA was 100 A_{260}, *E. coli* t-RNA was 81 A_{260}. Incubation was at 37°. At the indicated times, 0.4-ml aliquots were withdrawn and processed as in Table I.

et al. (1951). Chromatography on methylated albumin kieselguhr (Mandell and Hershey, 1960) was carried out as described previously (Lazzarini and Peterkofsky, 1965). The amino acid dependent ATP–PP_i exchange assay was essentially as described by Berg *et al.* (1961). The esterification of leucine or other amino acids to t-RNA by trapping on millipore filters was measured by a previously described method (Peterkofsky, 1964).

Results

Initial attempts to determine satisfactory conditions under which leucine might be acylated to *E. coli* t-RNA by the aminoacyl-t-RNA synthetase from yeast showed an unusual variability in the maximum yield of acylated RNA in different experiments. It soon became obvious that, while acylation of leucine to *E. coli* t-RNA by the enzyme from *E. coli* (homologous reaction) was essentially insensitive to changes in the assay conditions, the maximum leucine acceptance of *E. coli* t-RNA catalyzed by the heterologous yeast enzyme was markedly susceptible to variations in the reaction conditions (Table I). The following series of experiments were designed to characterize this phenomenon.

A standard incubation mixture was adopted (GSH system of Table I). This gave the highest level of leucine incorporation to *E. coli* t-RNA with either the aminoacyl-t-RNA synthetase from *E. coli* or yeast (Peterkofsky, 1964). Under these conditions, the yeast enzyme catalyzed the attachment of leucine to *E. coli* t-RNA to about 70% the extent that the *E. coli* enzyme does. As shown in Figure 1, if leucine acylation incubation mixtures containing yeast enzyme and *E. coli* t-RNA are supplemented with NaCl, at concentrations above 0.05 M, there is a marked reduction in both the rate and the

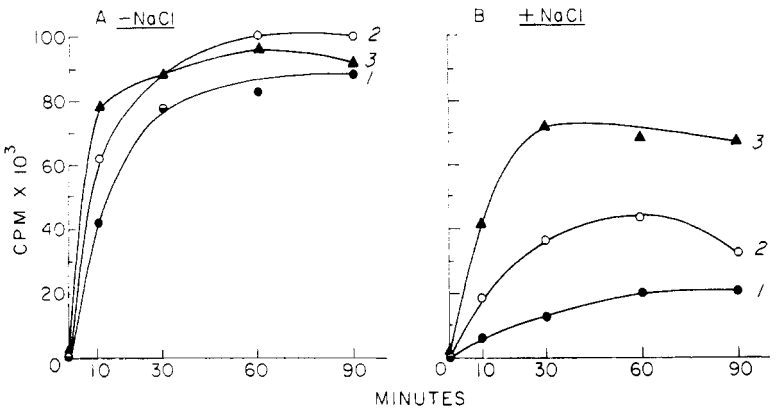

FIGURE 3: Effect of yeast leucyl-t-RNA synthetase concentration on leucine acylation to *E. coli* t-RNA in the presence and absence of NaCl. Incubation mixtures were prepared as for Figure 2. Where indicated, NaCl was at a level of 0.12 M. The levels of yeast enzyme were: curve 1, 0.02 ml; curve 2, 0.06 ml; curve 3, 0.24 ml. Leucyl-t-RNA formation was measured in 0.4-ml aliquots as in Table I.

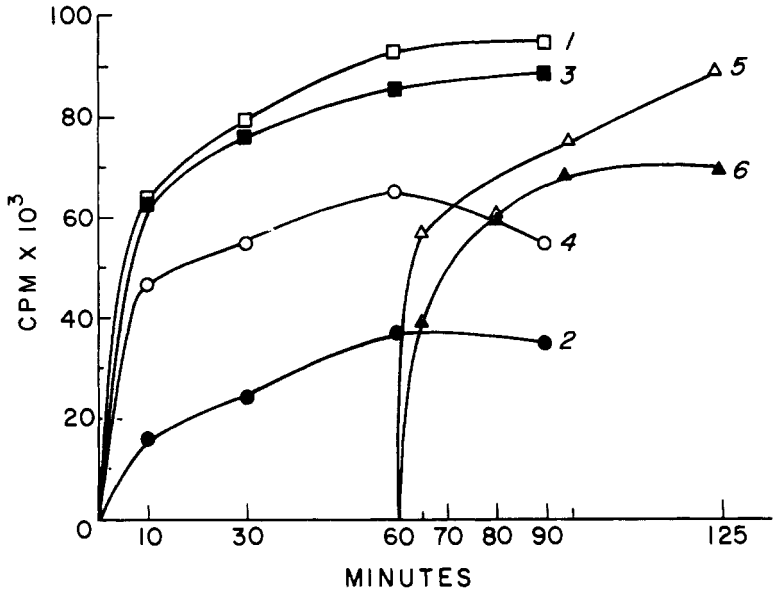

FIGURE 4: Effect of preincubation in the absence of yeast leucyl-t-RNA synthetase on the inhibition by NaCl of leucine acylation to *E. coli* t-RNA. Incubation mixtures were prepared as for Figure 2. In incubations 2, 4, and 6, NaCl was at a level of 0.12 M. Incubations 1 and 2 contained 0.06 ml of enzyme; incubations 3–6 contained 0.24 ml of enzyme. In incubations 5 and 6, the enzyme was added to all the other components of the incubation after 60 min of preincubation. At the specified times, 0.4-ml aliquots were withdrawn and treated as in Table I.

extent of the acylation reaction. Control incubations with the *E. coli* synthetase showed no such inhibition and suggested that this behavior was unique to the yeast enzyme.

The data of Figure 2 show that the addition of NaCl (0.12 M) has essentially no effect in incubation mixtures where yeast enzyme and yeast t-RNA (A-1) or *E. coli* enzyme and *E. coli* t-RNA (B-1) are together. However, there appears to be a marked effect of NaCl in reducing the rate of formation and yield of leucyl-t-RNA where yeast enzyme and *E. coli* RNA (A-2) or *E. coli* enzyme and yeast RNA (B-2) are combined. As previously pointed out by Yamane and Sueoka (1963) the attachment of leucine to yeast t-RNA by *E. coli* enzyme occurs to only a small extent (note that the incorporation scale in B-2 is lower than that in the other curves by a factor of approximately 10). Furthermore, it may be that, under these circumstances, the leucine does not become attached to one of the normal species of leucine acceptor RNA. In the case of the cross with yeast enzyme and *E. coli* RNA, the addition of NaCl depresses the yield of leucyl-t-RNA

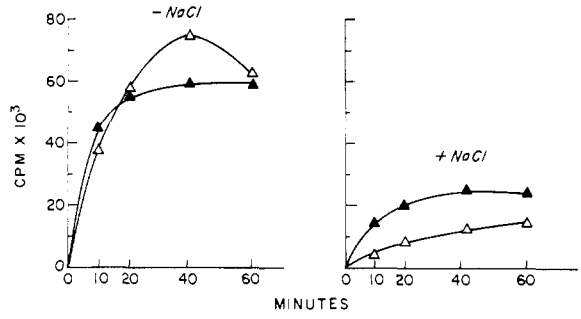

FIGURE 5: Effect of concentration of preincubated yeast leucyl-t-RNA synthetase on the inhibition by NaCl of leucine acylation to *E. coli* t-RNA. Incubation mixtures were prepared as for Figure 2. NaCl, where added, was at 0.12 M. The incubation mixtures were made complete with the exception of t-RNA and incubated for 60 min at 37°. The acylation reaction was then initiated by the addition of t-RNA. △, 0.06 ml of enzyme; ▲, 0.24 ml of enzyme. At the specified times, 0.4-ml aliquots were withdrawn and treated as in Table I.

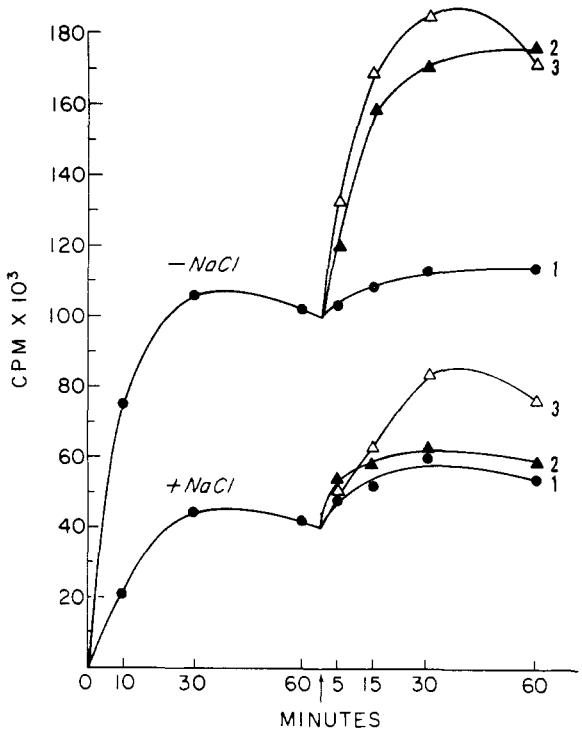

FIGURE 6: Effect of readdition of enzyme, t-RNA, or both after the acylation of leucine to *E. coli* t-RNA by yeast leucyl-t-RNA synthetase has gone to completion. Incubation mixtures were prepared as for Figure 2. NaCl, where added, was at 0.12 M. The mixtures contained 0.025 ml of yeast enzyme/ml. After incubation at 37° for 60 min, the following additions were made to the incubation mixtures: ●, an aliquot of enzyme equivalent to the original addition, ▲, an aliquot of t-RNA equivalent to the original addition, △, an aliquot of both enzyme and t-RNA equivalent to the original additions. At the specified times, 0.4-ml aliquots were withdrawn and treated as in Table I.

to about one-third of that in the control. However, in the case of the cross between *E. coli* enzyme and yeast t-RNA, it is not clear whether the addition of NaCl leads to a change in the final yield of leucyl-t-RNA although there is clearly a difference in the rate of acylation. Since the low level of reaction in that cross-reacting system makes reliable studies difficult, no further studies were conducted on the attachment of leucine to yeast t-RNA by the *E. coli* enzyme.

Figure 3 describes the effect of yeast enzyme concentration on the kinetics of attachment of leucine to *E. coli* t-RNA. In the absence of added NaCl (Figure 3A), the rate of leucine acylation was proportional to the level of enzyme while the yield of leucyl-t-RNA was independent of the level of enzyme. However when the standard incubation mixture was supplemented with NaCl (Figure 3B), both the rate and the *yield* of leucyl-t-RNA became dependent on the concentration of the enzyme. These data showed that the inhibition by NaCl of the heterologous leucine acylation reaction observed in the standard incubation system was reversed by a high concentration of enzyme.

One possible interpretation of the results in Figure 3 is that, under the influence of NaCl, *E. coli* t-RNA undergoes a slow structural modification (Boedtker, 1960) to a form that cannot be acylated by the yeast activating enzyme. The experiment detailed in Figure 4 presents evidence against this possibility. In agreement with the data of Figure 3, it is seen that the extent of leucyl-t-RNA formation is identical at two levels of enzyme in the absence of NaCl (curves 1 and 3). In the presence of NaCl, however, the rate and yield of leucyl-t-RNA are depressed, but not equally at two different levels of enzyme (curves 2 and 4). It was reasoned that, if the t-RNA is undergoing modification during the course of the reaction, preincubation of the reaction components in the absence of the enzyme would eliminate the capacity of the higher level of enzyme to overcome the inhibition by salt. The data show, however, that the preincubation appears not to affect the inhibition of the acylation reaction (curves 5 and 6). Thus, it appeared more probable that it was the enzyme rather than the RNA or some other component of the incubation that was undergoing modification in the presence of NaCl.

Figure 5 shows the effect of enzyme concentration on the acylation reaction using enzyme preincubated in the absence or presence of NaCl. Comparison with the data of Figure 4 shows that without preincubation of the enzyme, there was about a 60% inhibition of leucyl-t-RNA formation due to NaCl at the low level of enzyme and about 25% inhibition due to NaCl at the higher level of enzyme. However, when the

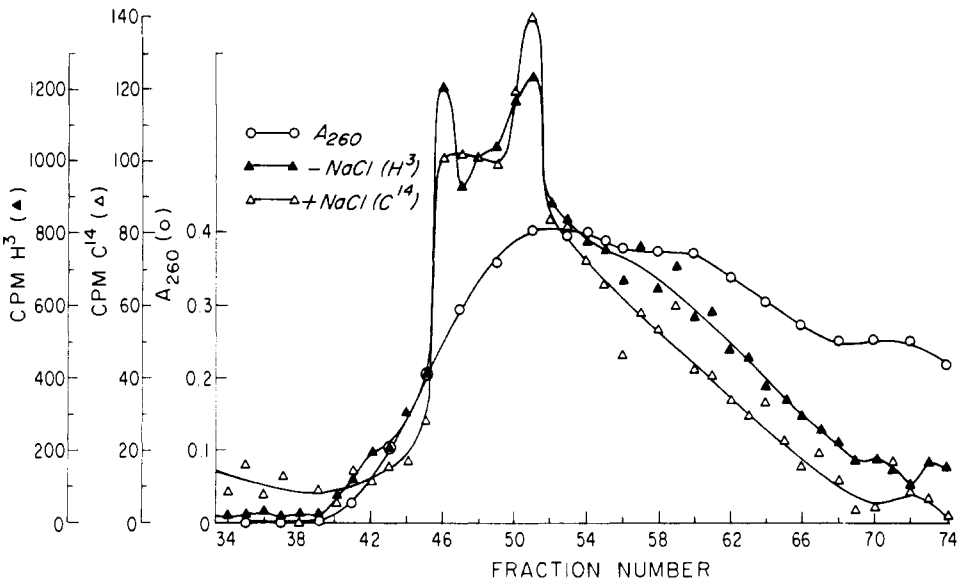

FIGURE 7: Methylated albumin kieselguhr column elution profile of *E. coli* t-RNA acylated by yeast leucyl-t-RNA synthetase in the presence or absence of NaCl. *E. coli* t-RNA (40 A_{260}) was incubated with yeast leucyl-t-RNA synthetase as in Figure 2. One incubation mixture contained NaCl (0.12 M) and [^{14}C]leucine. A second, otherwise identical mixture, contained no NaCl, but [^{3}H]leucine. The reaction mixtures, complete except for the t-RNA, were incubated for 60 min at 37°. The t-RNA was then added and samples of the incubation mixture were withdrawn every 15 min to check the extent of acylation. When the t-RNA was maximally acylated, the incubation mixtures were shaken with an equal volume of 88% phenol. The s-RNA was precipitated from the aqueous phase by the addition of 0.1 volume of 20% potassium acetate and 2 volumes of ethanol. The leucyl-t-RNA was dissolved in and dialyzed overnight against 0.005 M potassium cacodylate, pH 5.5. A mixture was made of 9.6 A_{260} of [^{3}H]leucine t-RNA containing approximately 76,000 cpm and 30 A_{260} of [^{14}C]leucine t-RNA containing approximately 6800 cpm. The mixture was fractionated by MAK column chromatography. Fractions (2 ml) were collected. Bovine serum albumin (0.2 mg) was added to each fraction, which was then precipitated by TCA (5%) and trapped on Millipore filters. The filters were dissolved in scintillation fluid and counted at appropriate settings for double-labeled counting in a Packard scintillation spectrometer.

enzyme was preincubated (Figure 5), the inhibition of leucyl-t-RNA formation due to NaCl increased to about 80% at the low level of enzyme and 60% at the high level of enzyme. These data indicated that it was indeed the enzyme that was modified by exposure to NaCl.

Figure 6 shows the difference in acylation kinetics of reaction mixtures with and without NaCl when additional incubation components are added at a second stage. In the first stage, it is seen that in the presence of NaCl, only 40% of the normal level of leucyl-t-RNA is formed. When the reaction mixture not supplemented with NaCl has an additional amount of enzyme added to it at 60 min (curve 1), no further acylation occurs, indicating that the t-RNA is fully acylated. However, when an additional amount of RNA equivalent to the original amount in the incubation mixture is added (curve 2) more leucine acylation takes place, indicating that the enzyme is still active. A somewhat different situation applies when later additions are made to the incubation mixture containing NaCl. The addition of a further aliquot of enzyme (curve 1) results in only a small further acylation such that the t-RNA is still only 50% as well charged with leucine as is RNA not exposed to NaCl. Furthermore, the addition of a further aliquot of acceptor RNA (curve 2) leads to only a minor increase in the level of aminoacyl-t-RNA. These data support the notion that in the presence of enzyme and NaCl, both the enzyme and t-RNA lose a portion of their activity. A generalized reaction inhibitor apparently is not formed during the course of the reaction, since a supplemental addition of both *E. coli* t-RNA and yeast enzyme (curve 3) leads to a doubling of the amount of leucyl-t-RNA, at the expected salt-inhibited level.

E. coli t-RNA is characterized by multiple species of leucine t-RNA which can be partially resolved by chromatography on methylated albumin (Yamane and Sueoka, 1963). Figure 7 was an attempt to determine whether the lower yield of leucyl-t-RNA formed in acylation reactions containing NaCl was due to complete charging of only one of the subspecies of leucine acceptor RNA or alternatively to incomplete charging of all the RNA species that are normally acylated by the yeast synthetase. *E. coli* t-RNA was

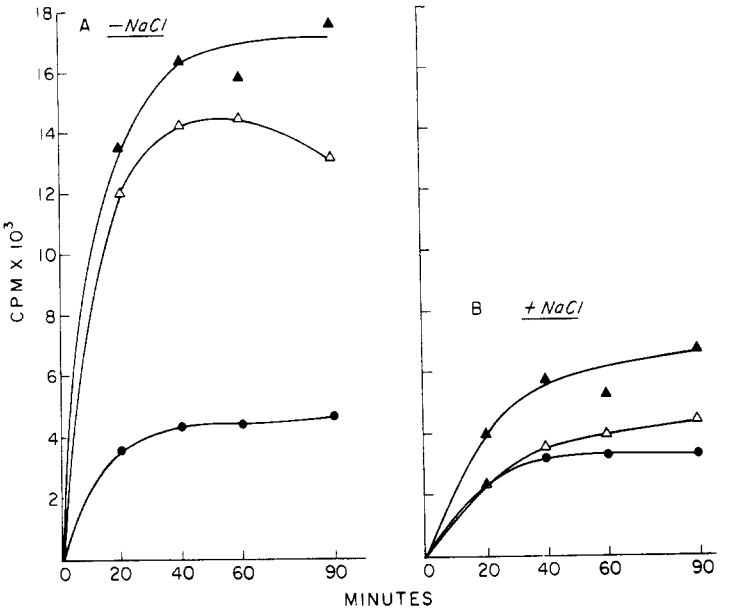

FIGURE 8: Effect of yeast t-RNA on the inhibition by NaCl of the acylation of leucine to *E. coli* t-RNA by yeast leucyl-t-RNA synthetase. Incubation mixtures were prepared as for Figure 2, containing yeast enzyme, and were complete except for the t-RNA. After preincubation at 37° for 60 min, the t-RNA was added. Yeast t-RNA was added at a level of 0.71 A_{260}/ml, *E. coli* t-RNA at a level of 2.75 A_{260}/ml. ●, yeast t-RNA alone; △, *E. coli* t-RNA alone; ▲, both yeast t-RNA and *E. coli* t-RNA. At the indicated times, 0.4-ml aliquots were withdrawn and processed as in Table I for determination of the extent of acylation.

charged with leucine by the yeast aminoacyl-t-RNA synthetase in the presence ([^{14}C]leucine) or absence ([^{3}H]leucine) of 0.12 M NaCl. There was about 65% inhibition of the maximal yield of leucine attached to RNA in the incubation mixture preincubated with NaCl. After reisolation, the t-RNA samples were combined and fractionated by chromatography on methylated albumin on kieselguhr (Mandell and Hershey, 1960). The elution profiles shown in Figure 7 indicate that the distribution of radioactivity associated with the leucyl-t-RNA formed in the presence or absence of NaCl is quite similar. This fractionation procedure does not provide a complete resolution of the different subspecies of leucine t-RNA (probably 3) that can be acylated with the yeast enzyme. These data, nevertheless, suggest that NaCl does not preferentially suppress the attachment of leucine to a particular species of leucine acceptor RNA.

An experiment was carried out to determine whether the inhibition of charging of *E. coli* t-RNA by the yeast enzyme could be reversed by yeast t-RNA (Figure 8). The data show that NaCl affects the level of acylation to *E. coli* t-RNA but not to yeast t-RNA. When t-RNA from both sources are included in the same incubation mixture, the level of leucine bound to t-RNA is the sum of that bound to the individual t-RNA preparations at the level characteristic of the presence or absence of NaCl. It is clear from this experiment that the addition of yeast t-RNA does not overcome the NaCl-induced inhibition of charging of *E. coli* t-RNA by the yeast enzyme. Thus yeast t-RNA does not have the property of reactivating an inhibited enzyme.

The following experiment established that the inhibition of the yeast enzyme due to NaCl is reversible by dialysis. As shown in Figure 9, the rate and extent of leucine attachment to *E. coli* t-RNA was compared with samples of yeast enzyme treated in different ways. Enzyme preincubated with NaCl (curve 2) showed approximately 75% inhibition of the amount of leucine bound to *E. coli* t-RNA by yeast enzyme which was preincubated in the absence of NaCl (curve 1). In one set of samples, after preincubation in the presence or absence of salt, the enzyme was dialyzed before testing. In this case, both the enzyme which had been exposed to NaCl (curve 4) and the sample which had not been exposed to NaCl (curve 3) showed the same capacity to maximally charge the *E. coli* t-RNA. This experiment therefore establishes that the change induced in the yeast enzyme by NaCl is reversible by dialysis.

The effect of NaCl on the leucine-dependent ATP–pyrophosphate exchange reaction catalyzed by the yeast leucyl-t-RNA synthetase was investigated. The curves in Figure 10 show that there is essentially no inhibition by NaCl of the exchange reaction catalyzed by the yeast enzyme.

An attempt was made to correlate the unique NaCl-induced inhibition of the *E. coli* or yeast enzymes with heterologous t-RNA with changes in the kinetic constants for t-RNA (Figures 11–13). The effect of con-

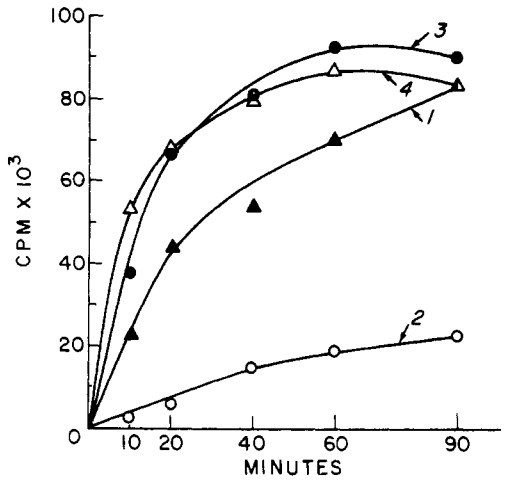

FIGURE 9: Reversibility by dialysis of the inhibition by NaCl of the acylation of leucine to *E. coli* t-RNA by yeast leucyl-t-RNA synthetase. Yeast leucyl-t-RNA synthetase was incubated for 60 min at 37° with (1 and 3) cacodylate buffer alone, (2 and 4) cacodylate buffer and 0.12 M NaCl. Samples 3 and 4 were then dialyzed for 4 hr against 0.025 M phosphate, pH 7.0. The extent of acylation of *E. coli* t-RNA was then determined as for Figure 2 with the different preparations of yeast enzyme.

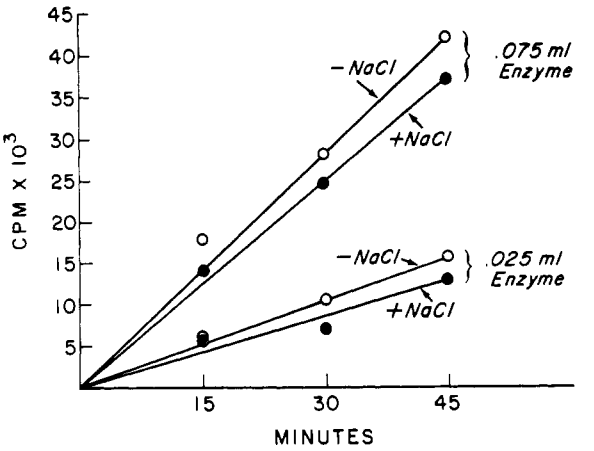

FIGURE 10: Effect of NaCl on the leucine-dependent pyrophosphate–ATP exchange catalyzed by yeast leucyl-t-RNA synthetase. In a total volume of 3.15 ml were added: potassium cacodylate, pH 7.0, 50 μmoles; GSH, 35 μmoles; $MgCl_2$, 35 μmoles; ATP, 20 μmoles; leucine, 1 μmole; and yeast enzyme, 0.025 ml or 0.075 ml. One set of incubations at each level of enzyme was supplemented with NaCl (0.12 M). Control incubations lacking leucine were set up for each level of enzyme with and without NaCl. The incubation mixtures were preincubated for 60 min at 37°, then in a total volume of 1.85 ml were added: sodium fluoride, 300 μmoles; and sodium pyrophosphate 20 μmoles containing approximately 2×10^6 cpm. At the indicated times, 1-ml aliquots were treated with 1 ml of 7% perchloric acid in 0.2 M $Na_4P_2O_7$. NaCl (0.12 ml, 1 M) was then added to those aliquots not supplemented with NaCl. A suspension (1.0 ml) of acid-washed Norit containing 30 mg/ml was then added. The contents of the test tubes were collected on Millipore filters and washed with three 20-ml portions of distilled water. The filters were inverted on planchets and counted in a Nuclear-Chicago thin-window gas-flow counter. The counts presented are corrected for that obtained in the incubation mixtures lacking leucine.

centration of acceptor RNA on the kinetics of the acylation reaction was measured in the presence and absence of NaCl (0.12 M). Figure 11 shows a plot of the rate of leucine acylation to *E. coli* t-RNA by the *E. coli* enzyme as a function of t-RNA concentration in the presence and absence of NaCl. There are several noteworthy features that become apparent from this plot. There is approximately a 70% inhibition of the V_{max} due to NaCl. At higher concentrations of t-RNA, the velocity in the absence of NaCl decreases to a value that approaches the velocity seen in the presence of NaCl. It might also be pointed out that in the absence of NaCl, the *v vs. s* plot shows somewhat of a sigmoidal character, suggesting that there may be a cooperative or allosteric effect of the t-RNA. Because of these features of the kinetics, analysis by reciprocal plots was not attempted. Nevertheless, it appears that there is no gross change in the K_m for t-RNA in the presence of NaCl. It may be estimated that the K_m for *E. coli* leucine acceptor RNA for the *E. coli* synthetase is about 2.5×10^{-8} M. This is approximately the range that has been reported for other amino acid activating enzymes (Calendar and Berg, 1965; Makman and Cantoni, 1965; Baldwin and Berg, 1966).

Figure 12 shows the *v vs. s* plot for leucine acylation in the yeast enzyme–yeast t-RNA pair. The most obvious difference here compared to Figure 11 is that there is no effect on the kinetic parameters (either K_m or V_{max}) as a result of the salt addition. The two unusual kinetic features that were seen with the *E. coli* t-RNA are also seen here. The curve is sigmoid in shape and the velocity does not approach a stable saturation value, but decreases at higher substrate concentrations. A rough estimate of the K_m for yeast leucine acceptor RNA for the yeast activating enzyme is 4×10^{-7} M.

As might have been expected, the most marked change in kinetics due to NaCl is in the heterologous reaction of the yeast synthetase and *E. coli* t-RNA (Figure 13). Here it can be seen that there is approximately a tenfold change in the V_{max} due to NaCl. It may be estimated that the K_m for *E. coli* leucine acceptor RNA for the yeast synthetase is about 1.6×10^{-7} M. This shows that *E. coli* leucine t-RNA has an affinity for the *E. coli* synthetase about six times better than for the heterologous yeast enzyme. It might also be noted that in the heterologous reaction, there is no indication for a decrease in the velocity at the higher RNA concentrations.

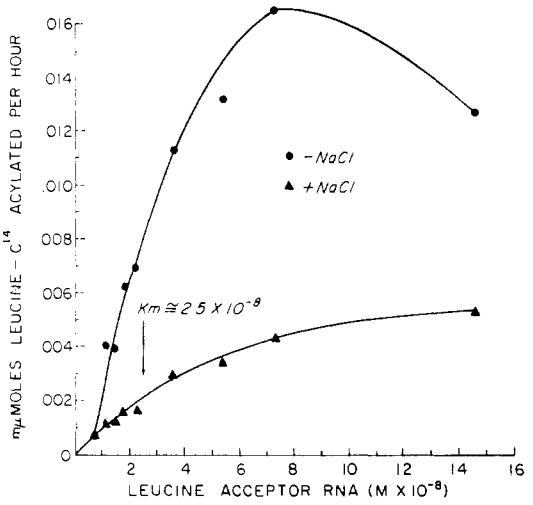

FIGURE 11: Effect of concentration of *E. coli* t-RNA on the rate of [^{14}C]leucine acylation by *E. coli* leucyl-t-RNA synthetase in the presence and absence of NaCl. Incubation mixtures in a total volume of 2 ml contained: potassium cacodylate, pH 7.0, 50 μmoles; ATP, 20 μmoles; $MgCl_2$, 35 μmoles; GSH, 35 μmoles; and *E. coli* enzyme, 0.0025 ml. One set of incubation mixtures contained 0.12 M NaCl. After preincubation at 37° for 60 min, 0.008 μmoles of [^{14}C]leucine (220 mc/mmole) and the indicated amounts of *E. coli* t-RNA were added. The concentration of leucine acceptor RNA in the t-RNA is calculated from the maximum acylation of [^{14}C]leucine attained with excess *E. coli* leucyl-t-RNA synthetase. Aliquots of the incubation mixtures were removed at 10-min intervals and precipitated with TCA for measurement of [^{14}C]leucine acylation as in Table I. The initial rate of acylation was calculated from these data and expressed as micromoles of [^{14}C]leucine acylated per hour.

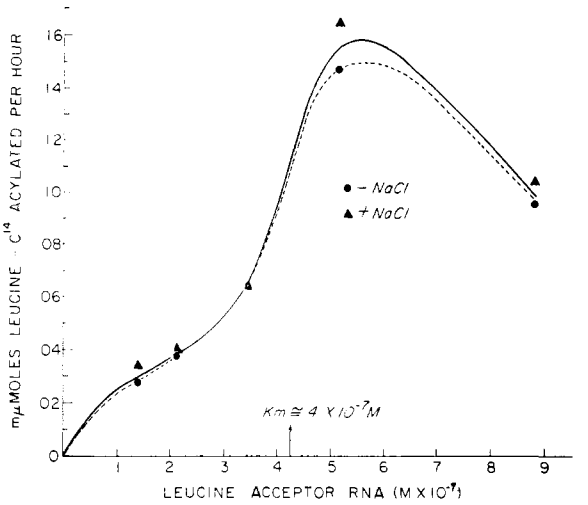

FIGURE 12: Effect of concentration of yeast t-RNA on the rate of [^{14}C]leucine acylation by yeast leucyl-t-RNA synthetase in the presence and absence of NaCl. Incubation conditions were exactly as in Figure 11, except that 0.03 ml of yeast enzyme and varying concentrations of yeast t-RNA were used. The concentration of leucine acceptor RNA in the t-RNA was calculated from the maximum acylation of [^{14}C]-leucine attained with excess yeast leucyl-t-RNA synthetase.

An experiment was carried out to determine whether the inhibition by NaCl of the attachment of leucine to *E. coli* t-RNA catalyzed by the yeast enzyme was unique for leucine (Figure 14). It can be seen that both lysine and alanine show a similar inhibition by NaCl of the rate and probably extent of acylation of these species of t-RNA. Thus, it is probable that the inhibition by NaCl of the heterologous attachment of amino acids to t-RNA is a generalized phenomenon.

Discussion

Several investigators have measured the capacity of enzymes derived from *E. coli* or yeast to attach leucine to t-RNA from these sources in either homologous or cross reactions. While Rendi and Ochoa (1962) presented evidence that the yeast enzyme could not acylate leucine to *E. coli* t-RNA, Doctor and Mudd (1963) showed that the yeast enzyme could attach leucine to *E. coli* t-RNA to about 25% the extent of the homologous enzyme. The experiments of Bennett *et al.* (1963),

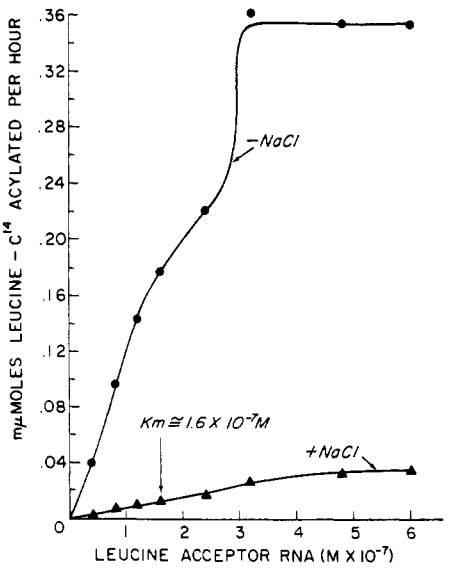

FIGURE 13: Effect of concentration of *E. coli* t-RNA on the rate of [^{14}C]leucine acylation by yeast leucyl-t-RNA synthetase in the presence and absence of NaCl. Incubations were exactly as in Figure 11, except that 0.02 ml of yeast enzyme and varying concentrations of *E. coli* t-RNA were used. The concentration of leucine acceptor RNA in the *E. coli* t-RNA was calculated from the maximum acylation of [^{14}C]leucine obtained with excess yeast leucyl-t-RNA synthetase.

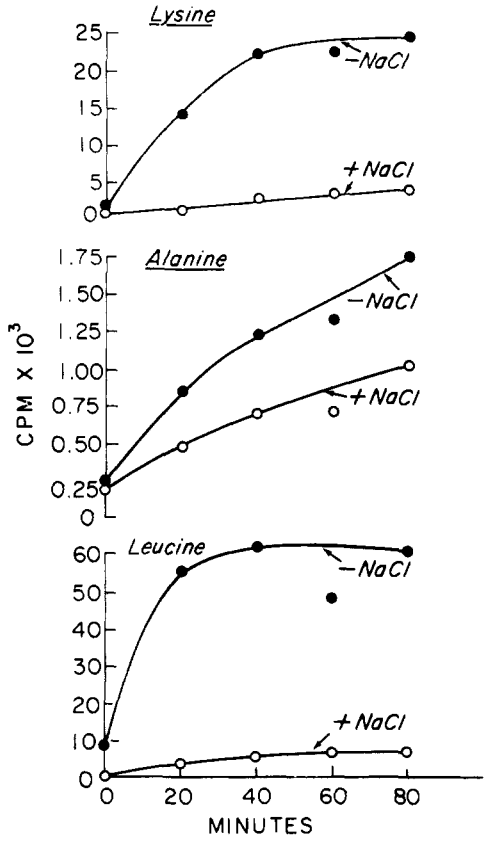

FIGURE 14: Effect of NaCl on the acylation of lysine, alanine, and leucine to *E. coli* t-RNA by yeast enzyme. Incubation mixtures, in a total volume of 1.2 ml, contained: potassium cacodylate, pH 7.0, 30 μmoles; ATP, 12 μmoles; $MgCl_2$, 20 μmoles; GSH, 21 μmoles; and yeast enzyme, 0.04 ml. Radioactive amino acid (0.005 μmole), either [^{14}C]lysine, [^{14}C]alanine, or [^{14}C]-leucine, was added to sets of reaction mixtures, one of which was supplemented with 0.2 M NaCl. After a preincubation of 45 min, 30 A_{260} of *E. coli* t-RNA was added. At the specified times, 0.2-ml aliquots were removed and assayed for aminoacylation.

Yamane and Sueoka (1963), and Lagerkvist and Waldenström (1964) indicated that the yeast leucyl-t-RNA synthetase could recognize 60–75% of the *E. coli* leucine t-RNA. The conditions used by the various investigators for measuring the esterification reaction vary markedly and it is difficult to point to a single factor to explain the various results. It is possible that the type of salt inhibition of heterologous aminoacylation described here contributes importantly to this variability. The indication that this type of inhibition is not unique to leucine suggests that surveys of heterologous amino acid esterification reactions may provide data which are markedly dependent on the assay conditions. Other factors, like sulfhydryl requirements (Bergmann *et al.*, 1961) or presence of nucleases in the t-RNA preparations, may also affect the results.

The work reported here has clearly not provided an adequate explanation at the molecular level for the effect of NaCl in depressing the yield of leucyl-t-RNA of *E. coli* formed by the yeast synthetase. The primary effect appears to be a reversible modification of the enzyme. However, the data of Figure 6 suggest that the salt-modified enzyme may interact with heterologous t-RNA, making them both unavailable for further reaction. It is worthy of note that a number of salts tested give an effect similar to the one described here for NaCl. NaCl, $(NH_4)_2SO_4$, and Tris–Cl appear to be more potent inhibitors than NH_4Cl, LiCl, or KCl, however.

The kinetic studies of Figure 2B indicated that there was essentially no effect of NaCl on the velocity of leucine acylation to *E. coli* t-RNA by the *E. coli* synthetase. However, it is apparent from the data of Figure 11 that, in the absence of NaCl, the velocity is dependent on t-RNA concentration. The maximum velocity occurs at a concentration of t-RNA approximately 20 times lower than that used in the experiment of Figure 2B. At higher concentrations of t-RNA, the velocity decreases. This probably explains why the data of Figure 2 show no marked difference in velocity in incubations with and without NaCl.

The work of Hele (1964), and Loftfield and Eigner (1965) and Hele and Barth (1966), has already provided a basis for the belief that aminoacyl-t-RNA synthetases may be "allosteric" proteins. The determination of the dependence on t-RNA concentration for the velocity of leucine esterification to t-RNA by the yeast or *E. coli* enzyme reported here lends some further support to this idea. Extensive studies have been conducted on the variation of RNA composition of bacteria as a function of growth rate (Neidhardt and Magasanik, 1960; Kjeldgaard and Kurland, 1963; Rosset *et al.*, 1964). If aminoacyl-t-RNA synthetases show allosteric stimulations as a result of variations in the concentration of t-RNA, this might add a greater dimension to the type of control of protein synthesis that may be exercised by t-RNA (Ames and Hartman, 1963; Stent, 1964).

References

Ames, B. N., and Hartman, P. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 349.

Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241*, 831.

Bennett, T. P., Goldstein, J., and Lipmann, F. (1963), *Proc. Natl. Acad. Sci. U. S. 49*, 850.

Berg, P. (1961), *Ann. Rev. Biochem. 30*, 293.

Berg, P., Bergmann, F. H., Ofengand, E. J., and Dieckmann, M. (1961), *J. Biol. Chem. 236*, 1726.

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

Boedtker, H. (1960), *J. Mol. Biol. 2*, 171.

Brown, G. L. (1963), *Progr. Nucleic Acid Res. 2*, 259.

Calendar, R., and Berg, P. (1965), *Federation Proc. 24*, 217.

Doctor, B. P., and Mudd, J. A. (1963), *J. Biol. Chem. 238*, 3677.

Goldstein, J., Bennett, T. P., and Craig, L. C. (1964),

Proc. Natl. Acad. Sci. U. S. 51, 119.
Hele, P. (1964), *Biochim. Biophys. Acta 87*, 449.
Hele, P., and Barth, P. T. (1966), *Biochim. Biophys. Acta 114*, 149.
Hoagland, M. B., Stephenson, M. L., Scott, J. F., Hecht, L. I., and Zamecnik, P. C. (1958), *J. Biol. Chem. 231*, 241.
Kjeldgaard, N. O., and Kurland, C. G. (1963), *J. Mol. Biol. 6*, 341.
Lagerkvist, U., and Waldenström, J. (1964), *J. Mol. Biol. 8*, 28.
Lazzarini, R. A., and Peterkofsky, A. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 549.
Loftfield, R. B., and Eigner, E. A. (1965), *J. Biol. Chem. 240*, PC1482.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Makman, M. H., and Cantoni, G. L. (1965), *Biochemistry 4*, 1434.
Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.
Muench, K. H., and Berg, P. (1966), *Biochemistry 5*, 970.
Neidhardt, F. C., and Magasanik, B. (1960), *Biochim. Biophys. Acta 42*, 99.
Peterkofsky, A. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1233.
Peterkofsky, A., Jesensky, C., Bank, A., and Mehler, A. H. (1964), *J. Biol. Chem. 239*, 2918.
Rendi, R., and Ochoa, S. (1962), *J. Biol. Chem. 237*, 3707.
Rosset, R., Monier, R., and Julien, J. (1964), *Biochem. Biophys. Res. Commun. 15*, 329.
Stent, G. S. (1964), *Science 144*, 816.
Yamane, T., and Sueoka, N. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 1093.
Yu, C., and Rappaport, H. P. (1966), *Biochim. Biophys. Acta* (in press).

The Reaction of Guanine Derivatives with 1,2-Dicarbonyl Compounds*

Robert Shapiro and John Hachmann†

ABSTRACT: The reaction product of glyoxal with guanosine has been isolated. On the basis of its spectral properties, it has been assigned a structure (I) in which a new ring is formed involving the 1 and N^2 positions of the guanine ring and both carbonyls of glyoxal. Ninhydrin has been found to react smoothly with guanine and guanosine, and analogous structures have been postulated for the products of these reactions. The ninhydrin–guanine adduct reacts with sodium metaperiodate to give a product identified as N^2-phthalonylguanine. While the glyoxal–guanosine and ninhydrin–guanosine adducts react with nitrous acid to give xanthosine, phthalonylguanine is resistant to nitrous acid. These reactions are of potential value as methods for modification of the guanine residues of the nucleic acids.

Aldehydes have shown particular promise as reagents for the modification of nucleic acids. A large amount of attention has been directed especially to the use of formaldehyde for this purpose. It is an antiviral agent, and readily reacts with and inactivates the ribonucleic acid (RNA) of tobacco mosaic virus (Fraenkel-Conrat, 1954; Staehlin, 1958). The reaction has been explored with a variety of nucleic acids. The site of reaction in each case is believed to be at the amino groups of those adenine, cytosine, and guanine residues that are not involved in hydrogen bonding. The linkages formed are labile and are readily broken upon dialysis (Staehlin, 1958; Haselkorn and Doty, 1961). This reaction has been used to prevent the renaturation of thermally denatured deoxyribonucleic acid (DNA) (Grossman *et al.*, 1961) and to study the secondary structure of soluble RNA (s-RNA) (Penniston and Doty, 1963a,b; Zubay and Marciello, 1963). The effect of formaldehyde upon the coding properties of synthetic polynucleotides has been investigated (Michelson and Grunberg-Manago, 1964). Formaldehyde has also been used to elaborate the structure of a complex between polyinosinic acid and protonated poly-

* From the Department of Chemistry, New York University, New York, New York 10003. *Received May 9, 1966.* This research was supported by grants from the U. S. Public Health Service (GM-11437-02 and GM-11437-03).

† National Science Foundation Cooperative Fellow, 1964–1965, Allied Chemical Fellow, 1965–1966.