*EXISTENCE OF TWO PHENYLALANYL-sRNA SYNTHETASES IN NEUROSPORA CRASSA**

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The nature of cross reactions between sRNA of one organism and aminoacyl-sRNA synthetase of another organism has previously been analyzed (Yamane, Cheng, and Sueoka;[1] Yamane and Sueoka[2]) by using methylated albumin column fractionation (Sueoka and Yamane[3]). Our previous studies revealed that the interspecific combination of sRNA and the synthetase resulted in the following patterns: (*a*) no cross reaction was observed; (*b*) the same profile was secured as that of normal aminoacyl-sRNA; (*c*) only some component or components of normal amino acid acceptor RNA were charged; (*d*) an entirely different profile was noted. The first three situations are the normal cases, while the last situation is exceptional and has been encountered so far only in two instances. One is found in the formation of yeast leucyl-sRNA by *E. coli* enzyme (Yamane *et al.*,[1] Yamane and Sueoka[2]), and the other is the finding of Barnett and Jacobson[4] for *E. coli* phenylalanyl-sRNA by Neurospora enzyme. Further studies of the first case were hampered by the fact that the amount of yeast leucyl-sRNA formed by *E. coli* enzyme was only 1 per cent of the normal yeast leucyl-sRNA.[2] Barnett and Jacobson's finding on Neurospora–*E. coli* combination[4] is, however, quite serious in that the novel products are in large quantity. Outside of these two exceptions, the pattern of cross reactions indicates a strong tendency toward conserving the specificity between sRNA and aminoacyl-sRNA synthetase among entirely different classes of organisms. Further evidence supporting the conservation has been accumulated.[5]

The present investigation was undertaken to settle the question of whether or not the charging of phenylalanine by Neurospora enzyme to several different sRNA's of *E. coli* is catalyzed by one and the same enzyme. If it is, this is a clear violation of the conservation of interspecific cross reaction between sRNA and the synthetase. The present investigation reveals that there are two enzymes in Neurospora which can activate phenylalanine. One attaches phenylalanine to the normal phenylalanine acceptor RNA of *E. coli*, while the other attaches phenylalanine to Neurospora sRNA and to the wrong sRNA's of *E. coli*.

Materials and Methods.—Strains: The following strains were used: *Neurospora crassa* wild-type strain 74A (kindly donated by Dr. Henry J. Vogel); yeast (a strain of baker's yeast); *Escherichia coli* B; *Pseudomonas aeruginosa* (American Type Culture Collection #10197); *Aerobacter aerogenes* (ATCC #9624).

Media: For normal mycelium *N. crassa* was grown on Beadle and Tatum minimal medium containing 2% sucrose.[6] For protoperithecia formation *N. crassa* was grown on agar plates of Westergaard and Mitchell minimal medium containing 2% sucrose.[7] Bacteria were grown as described in a previous report.[3]

Preparation of enzyme extract: Mycelium of Neurospora in the exponential phase of growth was collected on filter paper, washed once with cold 0.1 *M* Tris-HCl buffer (pH 7.3) containing 0.001 *M* Mg-acetate, and frozen in a deep freezer (−80°C). The frozen mycelium was ground with sea sand in a pestle and suspended in an equal volume of cold Tris-HCl buffer (pH 7.3) containing 0.001 *M* Mg-acetate. The extract was centrifuged at 12,100 × *g* for 10 min and the supernatant removed and centrifuged at 105,000 × *g* for 180 min. The supernatant was removed

and dialyzed against 0.01 *M* Tris-HCl buffer (pH 7.3) containing 0.001 *M* Mg-acetate and 0.0035 *M* mercaptoethanol in the cold overnight. The crude extract was applied to a DEAE cellulose column and fractionated. The enzyme fraction of *Neurospora crassa* was prepared free from RNA by passing the crude extract through a DEAE cellulose column using the same method as for *E. coli* B.[2]

Preparation of sRNA and aminoacyl-sRNA: The sRNA was prepared by the phenol procedure described by von Ehrenstein and Lipmann.[8] sRNA was prepared from Neurospora ascospore (kindly donated by Dr. Roger Storck) by grinding with three times their weight of sea sand in liquid nitrogen in a pestle, extracting with 20 vol of 0.001 *M* Tris-HCl buffer (pH 7.3) containing 0.01 *M* Mg-acetate, and shaking the extract with phenol. sRNA's were charged with either H^3-DL-phenylalanine (675 $\mu c/\mu M$, Schwarz BioResearch, Inc.) or C^{14}-L-phenylalanine (334 $\mu c/\mu M$, New England Nuclear Corp.) in the following reaction mixture (1.0 ml): 100 μmoles of Tris-HCl buffer (pH 7.3); 50 μmoles of Mg-acetate; 2.6 μmoles of ATP; 4 μmoles of reduced glutathione; 0–1 mg of sRNA; 0.5–1.0 μc of labeled phenylalanine; an appropriate amount of enzyme extract. The reaction mixture was incubated at 37°C for 6–30 min. Aminoacyl-sRNA was isolated by the phenol procedure and fractionated on methylated albumin columns according to the method previously reported,[2] except that 0.05 *M* sodium phosphate buffer, pH 6.3, was used for the elution. The differential counting of C^{14} and H^3 was made in a Packard Tricarb liquid scintillation counter.

Results.—Cross reactions: The result of Barnett and Jacobson[4] has been reproduced in our laboratory. Their discovery is shown in Figure 1. The profile, however, is variable depending on the duration of incubation (Fig. 2); the longer incubation gives a relative decrease of peaks II and III. This apparent decrease is due to the enzymatic discharge of phenylalanine from once-formed phenylalanyl-sRNA (peaks II and III) in the present charging condition (see *Materials and Methods*). On the other hand, the peak I phenylalanyl-sRNA, identical to *E. coli* normal phenylalanyl-sRNA, is stable.

Another difference in the two phenylalanine charging reactions (one to peaks II and III, the other to peak I) was disclosed when polyvinyl sulfate (PVS) was added to the reaction mixture. In the presence of these compounds, the charging of peaks II and III was markedly inhibited, while the charging of peak I was hardly affected (Fig. 3). The presence of 125 μg/ml bentonite showed a similar effect.

Fractionation of Neurospora phenylalanyl-sRNA synthetase: Crude enzyme preparation from growing Neurospora was fractionated on a DEAE cellulose column by both a stepwise and a gradient increase of an NaCl concentration (Figs. 4*A* and *B*). The relative amount of protein in each fraction was indicated by absorption at 280 mμ. Phenylalanine charging activity was assayed in two ways in Figure 4*A*, using *E. coli* sRNA and Neurospora sRNA. The presence of two distinct phenylalanyl-sRNA synthetases is clear.

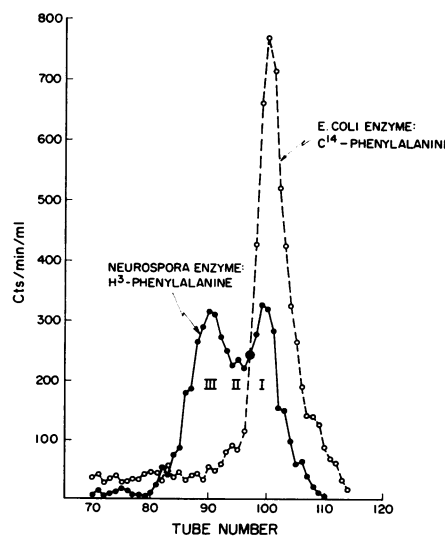


FIG. 1.—Elution profiles of *E. coli* phenylalanyl-sRNA charged by Neurospora enzyme (from Barnett and Jacobson[4]). "*E. coli* sRNA was charged with C^{14}-phenylalanine by the *E. coli* enzyme, reisolated, and combined with *E. coli* sRNA charged with H^3-phenylalanine by the Neurospora enzyme. The two sRNA's were then cochromatographed on methylated albumin. . . . For identification, three peaks in the heterologously chargeds RNA's profile have been designated I, II, and III."

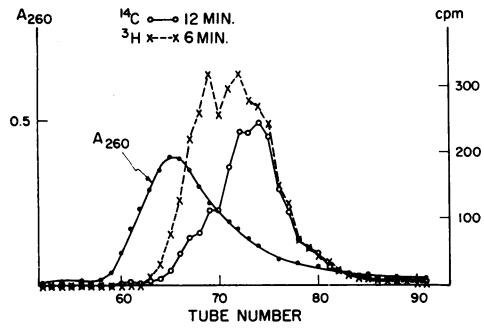


FIG. 2.—Comparison of the chromatographic profiles of *E. coli* H^3-phenylalanyl-sRNA and *E. coli* C^{14}-phenylalanyl-sRNA obtained by charging for 6 min or 12 min, respectively, at 37°C with Neurospora crude enzyme.

Although both of them charged phenylalanine to *E. coli* sRNA, the one eluting earlier (E1) did not charge Neurospora sRNA, while the one eluting later charged phenylalanine to both *E. coli* sRNA and Neurospora sRNA. The same situation is found also in the NaCl gradient elution (Fig. 4*B*). The result further shows that the E1 preparation is free from E2, while the E2 peak may contain some E1. Using PVS which inhibits E2 activity selectively (see the next section), contaminating E1 activity was demonstrated in the 0.15 *M* NaCl fraction, while little E1 activity was found in the 0.25 *M* NaCl fraction.

Characterization of E1 and E2: Differential attachments of phenylalanine to *E. coli* sRNA by the two enzymes are clearly shown in Figures 5*A* and *B*. Thus, E1 attaches phenylalanine to normal *E. coli* phenylalanine acceptor RNA, and E2 to abnormal sRNA (peaks II and III). The result of Figure 5*C* shows more clearly that the Neurospora E1 enzyme charges only normal *E. coli* phenylalanine acceptor RNA. When Neurospora sRNA was used as the substrate, E2 charged phenylalanine, while E1 hardly charged (Fig. 6*A*). This conclusion is also supported by the fact that the profiles of Neurospora phenylalanyl-sRNA either charged by Neurospora crude enzyme or by fractionated E2 are identical (Fig. 6*B*). Table 1 shows that *E. coli* enzyme barely charges phenylalanine on Neurospora sRNA, and the product of the cross reaction is the normal Neurospora phenylalanyl-sRNA.

Growth stages of Neurospora: Neurospora sRNA which would respond to E1 was searched by using sRNA from various stages of the life cycle of Neurospora (Table 1). In all cases examined, phenylalanine was charged only by E2. The presence of E1 and E2 was also found in germinating conidia, young mycelia, and old (stationary) mycelia.

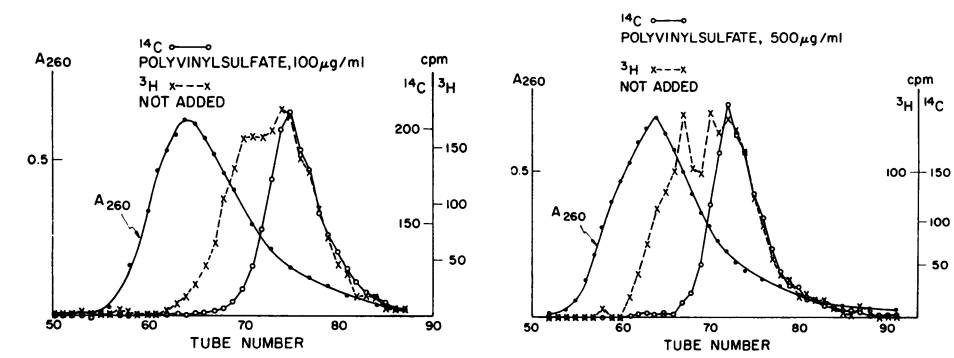
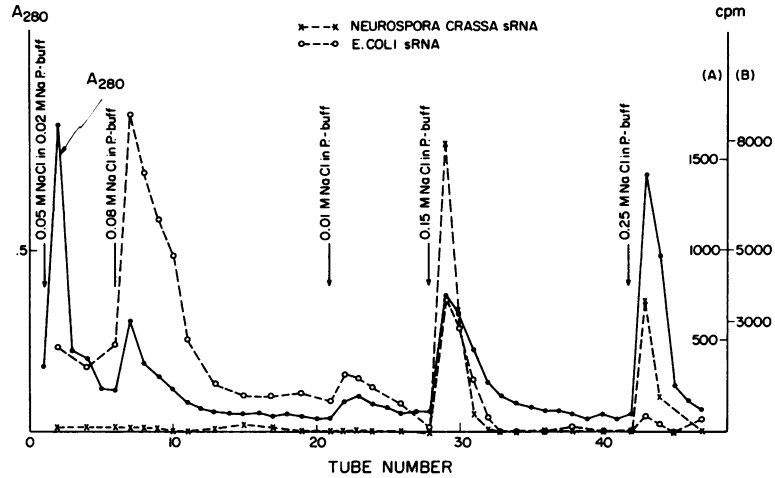
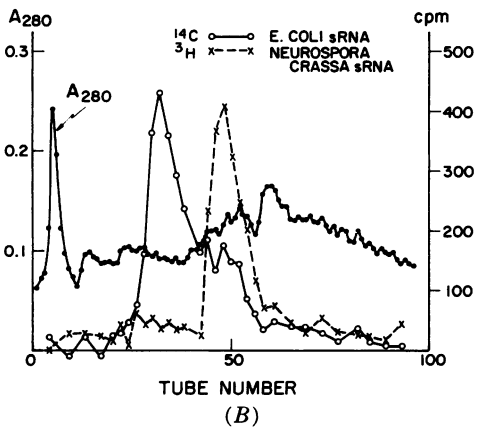


FIG. 3.—Effect of polyvinyl sulfate on phenylalanine activating enzymes. *E. coli* C^{14}-phenylalanyl-sRNA, obtained by charging for 6 min in the presence of 100 μg/ml (*A*) and 500 μg/ml (*B*) polyvinyl sulfate with Neurospora crude enzyme, was mixed with *E. coli* H^3-phenylalanyl-sRNA obtained under the same conditions, except for the addition of polyvinyl sulfate.



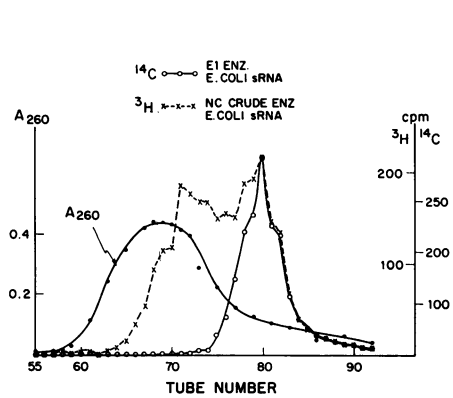
(A)



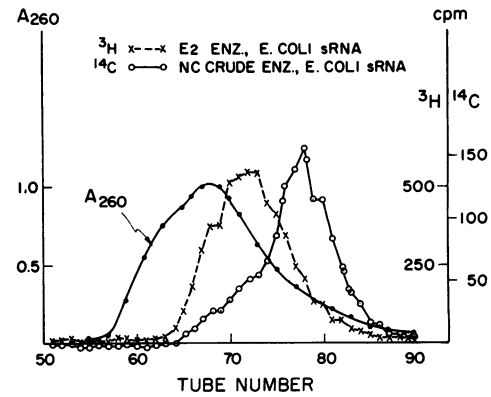
(B)

FIG. 4.—Elution profiles of Neurospora enzymes by DEAE cellulose chromatography. (*A*) DEAE cellulose column (1 × 5 cm) on which Neurospora crude enzyme (*ca.* 50 mg of protein) was applied was soaked with 20 ml of 0.02 *M* sodium phosphate buffer and then eluted with NaCl–0.02 *M* sodium phosphate buffer (pH 7.7). Four ml of eluate in each tube was collected. The enzyme activity was assayed in 0.25 ml of the reaction mixture containing 30 μg of *E. coli* or Neurospora sRNA, 0.06 ml of the enzyme fraction, and components described in *Materials and Methods*. After 15 min incubation, 0.2 mg of carrier DNA (crude salmon sperm) and 17 ml of 5% TCA were added to each tube. The precipitate was collected on the Millipore filter and counted. (*B*) *Neurospora crassa* crude enzyme preparation (*ca.* 86 mg of protein) was applied on a DEAE cellulose column (2 × 7 cm). Fractionation was carried out with a linear gradient of NaCl concentration (0–0.3 *M*) in 0.02 *M* sodium phosphate buffer (pH 7.7); 3.5 ml of eluate in each tube was collected. The assay procedure of enzyme activity was similar to that described in (*A*) except that the time of incubation was 10 min. The amount of radioactivity fixed on the filters without incubation at 37°C was subtracted in each value. The value was 53–110 cpm in each assay condition.

Range of cross reactions by E1 and E2: There is a clear demarcation in the pattern of cross reactions by E1 and E2 with sRNA of other organisms (Table 1). Interestingly enough, there is a definite tendency for E1 to cross-react with bacterial sRNA, and E2 with sRNA of higher organisms (Neurospora, yeast, and rabbit). The cross reaction of E2 with bacterial sRNA (although weak) may indicate a similar situation as in cross reaction between E2 and *E. coli* sRNA. This point is under investigation. The fact that only E2 charges phenylalanine to Neurospora sRNA is consistent with the tendency. A very weak cross reaction was, however, detected between *E. coli* enzyme and Neurospora sRNA for phenylalanine and the product of the cross reaction was similar to the normal Neurospora phenylalanyl-sRNA (Fig. 7). In this case, the rate of the cross reaction was about 1.5 per cent of the charging reaction with Neurospora enzyme and Neurospora sRNA.



(A)



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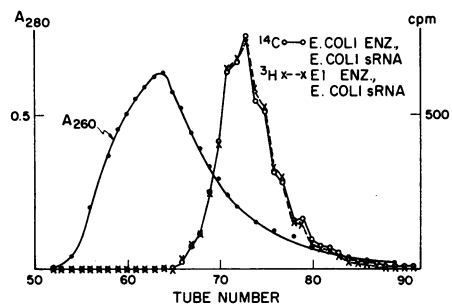
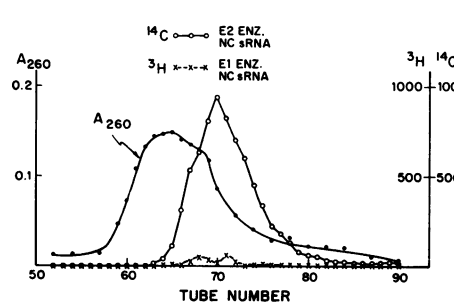


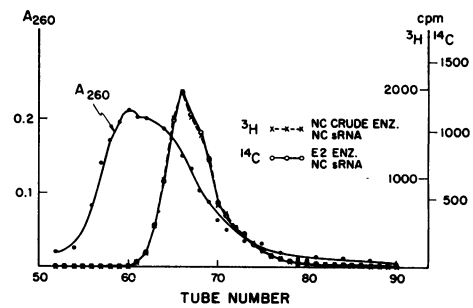
(C)

FIG. 5.—Comparison of the chromatographic profiles of (*A*) *E. coli* C^{14}–phenylalanyl-sRNA and *E. coli* H^{3}–phenylalanyl-sRNA obtained by charging for 6 min with Neurospora crude enzyme and fractionated enzyme (E1), respectively; (*B*) *E. coli* C^{14}–phenylalanyl-sRNA and *E. coli* H^{3}–phenylalanyl-sRNA obtained by charging for 12 min with *Neurospora crassa* crude enzyme and fractionated enzyme (E2), respectively; (*C*) *E. coli* C^{14}–phenylalanyl-sRNA and *E. coli* H^{3}–phenylalanyl-sRNA obtained by charging for 15 min with *E. coli* crude enzyme and Neurospora fractionated enzyme (E1), respectively.

Discussion.—The essential findings of the work are: (1) *Neurospora crassa* has two phenylalanyl-sRNA synthetases, E1 and E2, separable on a DEAE cellulose column; (2) E1 charges phenylalanine to *E. coli* phenylalanine acceptor sRNA, while E2 charges phenylalanine to two other *E. coli* sRNA's which normally do not accept phenylalanine; (3) Neurospora phenylalanyl-sRNA is formed by E2 but not by E1; (4) polyvinyl sulfate and bentonite inhibit E2 activity much more strongly than E1 activity; (5) both E1 and E2 are found in Neurospora mycelium of both the young and stationary stages while sRNA from no stage (vegetative mycelium,



(A)



(B)

FIG. 6.—Comparison of the chromatographic profiles of (*A*) Neurospora C^{14}–phenylalanyl-sRNA and Neurospora H^{3}–phenylalanyl-sRNA obtained by charging for 15 min with Neurospora fractionated enzymes, (E2) and (E1), respectively; (*B*) Neurospora C^{14}–phenylalanyl-sRNA and Neurospora H^{3}–phenylalanyl-sRNA obtained by charging for 15 min with Neurospora fractionated enzyme (E2) and Neurospora crude enzyme, respectively.

TABLE 1

ATTACHMENT OF PHENYLALANINE ONTO sRNA OF VARIOUS ORGANISMS BY THE TWO PHENYLALANYL-sRNA SYNTHETASES E1 AND E2

Source of sRNA		Enzyme		
	(μg)	E1 (3.1 μg)	E2 (6 μg)	*E. coli* crude (110 μg)
E. coli	(120)	1268 cpm	420 cpm	23,638 cpm
Ps. aeruginosa	(120)	996	327	10,464
A. aerogenes	(120)	398	106	5,956
N. crassa				
Growing hypha	(120)	0	5,965	360
Protoperithecia	(5)	35	804	77
Ascospore	(16)	12	3,202	199
Yeast	(120)	980	10,035	1,066
Rabbit liver	(60)	91	11,398	1,576

Assay of enzyme activity was carried out in 0.25 ml of the reaction mixture incubating for 10 min at 37°C. This is in the linear range of reaction. After incubation, labeled aminoacyl-sRNA was measured as described in the legend of Fig. 4*A*. The amount of radioactivity fixed on the filters without incubation at 37°C was subtracted in each value. The value was about 70 cpm in each assay condition.

ascospores, protoperithecia) can accept phenylalanine by E1; (6) E1 can charge phenylalanine to sRNA's of various bacteria and hardly charges sRNA's of Neurospora, yeast, and rabbit, while E2 weakly charges bacterial sRNA (e.g., some sRNA's of *E. coli* which do not normally accept phenylalanine) and charges sRNA's of Neurospora, yeast, and rabbit.

Judging from these facts, the existence of two phenylalanyl-sRNA synthetases in Neurospora could well mean that Neurospora retains in evolution a phenylalanyl-sRNA synthetase (E1) closer in origin to bacterial enzyme and that the other synthetase (E2) has a different origin or greatly altered enzyme which is closer to the phenylalanyl-sRNA synthetase of higher organisms. The unusual cross reaction between Neurospora E2 enzyme and *E. coli* sRNA, therefore, may come from fortuitous matching in stereospecificity between the enzyme recognizing site of some *E. coli* sRNA and the enzyme E2. This possibility has been raised for the abnormal cross reaction between yeast sRNA and *E. coli* enzyme for leucyl-sRNA formation.[9] An alternative interpretation is that the E1 enzyme can activate another amino acid as well as phenylalanine and in Neurospora the second amino acid can be charged on its own sRNA. In this connection, an activation of valine as well as isoleucine by *E. coli* isoleucyl-sRNA synthetase has been reported.[10] The above two possibilities are under experimental study.

The fact that *E. coli* enzyme can charge, even though slowly, phenylalanine to the normal Neurospora phenylalanine acceptor RNA (Table, 1 Fig. 7) indicates that conservation of sRNA versus synthetase specificity still exists between Neurospora and *E. coli*. In general there is, however, a large gap in the extent of conservation between bacteria and eucaryotic organisms (Yamane and Sueoka[5]). The func-

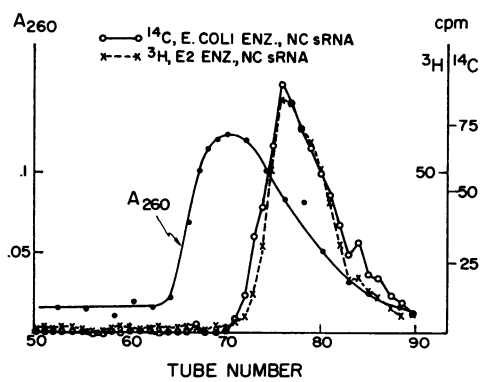


FIG. 7.—Comparison of chromatographic profile of Neurospora C^{14}-phenylalanyl-sRNA and Neurospora H^3-phenylalanyl-sRNA obtained by charging for 10 min with *E. coli* crude enzyme and with Neurospora fractionated enzyme (E2), respectively.

tional significance of E1 in Neurospora is not at all clear at the moment. It could be just an evolutionary remnant or it might be playing an important role in regulation of protein synthesis. Although sRNA accepting phenylalanine by E1 was not found in various stages of the Neurospora life cycle, a small amount may still exist throughout the life cycle or in a particular stage. Another possibility is that the E1 might be confined in some organelle in the cytoplasm.

Summary.—An unusual cross reaction between *Escherichia coli* sRNA and *Neurospora crassa* phenylalanyl-sRNA synthetase discovered by Barnett and Jacobson (1964) was found to be caused not by one enzyme but by two different enzymes, E1 and E2. E1 charges phenylalanine to normal *E. coli* phenylalanine acceptor RNA and does not charge Neurospora sRNA. On the other hand, E2 charges phenylalanine to other sRNA's of *E. coli* than normal phenylalanine acceptor RNA. E2, however, does charge phenylalanine to Neurospora sRNA. The difference between the two enzymes can be shown directly by DEAE cellulose fractionation and also by polyvinyl sulfate or bentonite inhibition of E2 enzyme activity but not E1 activity. We could not detect sRNA corresponding to E1 in various stages of the life cycle of Neurospora. The pattern of cross reactions by these two enzymes with sRNA's from various organisms indicates that E1 enzyme is similar to bacterial phenylalanyl-sRNA and E2 to higher organisms.

* We are grateful to Mrs. Carol Tornrose for her technical assistance. This work was supported by USPHS grant GM 10923-03 and National Science Foundation grant GB-1548.

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*INTERSPECIES AMINOACYL-sRNA FORMATION: FRACTIONATION OF NEUROSPORA ENZYMES INVOLVED IN ANOMALOUS AMINOACYLATION**

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The role of enzyme-aminoacyl-adenylates in protein synthesis has been well established (see review, ref. 1). These complexes interact selectively with amino acid–specific sRNA's, and their amino acid moiety is transferred into aminoacyl linkage with the RNA molecule. In light of the fact that subsequent codeword