We had found this same *Torpedo* preparation to have two binding sites for the agonists, muscarone and nicotine; the concentration of the site with highest affinity ranged between 0.06 to 0.08 nmole per gram of electroplax, and the one with lower affinity ranged between 0.4 to 0.8 nmole per gram of electroplax (*5*). Binding activity at both sites was partially destroyed by the above three enzymes. It is apparent that the concentrations of these sites and their enzyme sensitivity are similar for muscarone, nicotine, and acetylcholine. Binding in each case is antagonized by nonradioactive nicotinic ligands, it is reversible and of high affinity, and (at identical concentrations) it is not found with AChE. It is therefore suggested that the binding is to AChR.

On the basis of the characteristics of binding of muscarone and nicotine at both sites, we suggested that either the two sites are on the same macromolecule and are noninteracting or show negative cooperativity, or each site is carried by a different macromolecule, and the two could bear a relationship like that of isozymes to each other (*5*). By analogy, the same may be true for ACh. There have been several suggestions concerning the allosteric nature of AChR (*22*) and its existence in two conformations in vivo (*23*). Most proposals of protein conformational changes assume that the conformations are in rapid equilibrium; thus we would detect only an average of the two affinities under our experimental conditions. Consequently, it is a remote possibility that the two affinities for ACh presently detected or for other agonists represent the affinities of a single site in macromolecules existing in two conformations (*24*). Further identification and characterization awaits isolation of the binding macromolecules from this heterogeneous mixture.

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21. If it is assumed that the ACh molecule binds to the active site of the receptor by electrostatic (5 to 10 kcal), hydrogen (2 to 5 kcal), and hydrophobic (0.5 to 0.7 kcal) bonds, the average free energy supplied in the reaction would be 10.6 kcal/mole. Such energy is well within that required to give the high binding affinities observed at 4°C (—9.1 and —10.3 kcal/mole for the low and high affinities, respectively).
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24. *Note added in proof*: According to a newly proposed model [N. Laiken and G. Nemethy, *Biochemistry* **10**, 2101 (1971)], "flexible ligands cannot be expected to bind in accordance with the single-class model of the theory of multiple equilibria." The new model proposes that findings such as those described herein may reflect the binding of different ligand configurations to identical sites. As these sites are successively filled, subsequent ligand molecules ar restricted in the number of multifunctional attachments which they can form, and so bind with lesser affinity. This model offers another alternative for explaining the two binding affinities observed here.
25. We thank Dr. R. D. O'Brien for his encouragement and constructive criticism, Dr. T. Podleski for his helpful comments on this manuscript, and Dr. H. Howland for devising the computer program used. This research was supported by PHS grants NS 09144 and GM 07804 and training grant ES 98.

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Ribosome-Catalyzed Polyester Formation

Abstract. *Deamination of phenylalanyl–transfer RNA with nitrous acid yields the α-hydroxyacyl analog, phenyllactyl–transfer RNA. When this is incubated in a protein-synthesizing system directed by polyuridylic acid, it yields an acid-precipitable, alkali-labile polyester of phenyllactic acid.*

Peptidyl transferase is the enzyme in the ribosome which catalyzes the formation of peptide bonds during protein synthesis, and it is responsible for the biosynthesis of all proteins. In a study of the reactivity of this ribosomal enzyme we have demonstrated (*1*) that it can also catalyze the formation of ester bonds (*2*). Thus, for example, the ribosome can form an ester link between *N*-formylmethionine and the α-hydroxy analog of puromycin (*1*). We also have shown that ribosomes are able to insert α-hydroxy acids into protein through the use of α-hydroxyacyl-tRNA which is formed from aminoacyl-tRNA by treatment with nitrous acid (*3*). Hydroxy acids have been incorporated into a fragment of bacteriophage coat protein in an in vitro system with the use of viral RNA as a messenger for protein synthesis. A study of the hexapeptide fragment formed in this system showed that the ribosome would accept deaminated phenylalanyl- or alanyl-tRNA and incorporate them into the correct position in the polypeptide sequence, as determined by the position of the appropriate codon in the messenger RNA, with formation of an ester linkage in place of the normal peptide bond. However, this left open

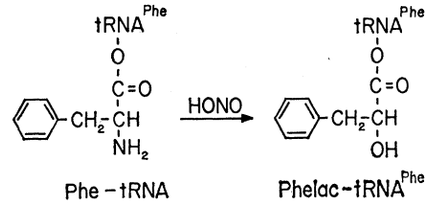

Fig. 1. Conversion of phenylalanyl-tRNA to phenyllactyl-tRNA. [^{14}C]Phelac-tRNA was prepared from [^{14}C]Phe-tRNA by nitrous acid deamination; 3 mg of [^{14}C]Phe-tRNA (*3*) was incubated in 0.25*M* sodium acetate, 0.01*M* magnesium acetate, 1*M* $NaNO_2$, at 24°C for 30 minutes on a Radiometer *p*H-stat maintained at *p*H 4.3 with 25 percent acetic acid. The RNA was then precipitated and washed with ethanol at −20°C. Analysis after alkaline hydrolysis revealed a residue of <0.1 to 0.5 percent phenylalanine. Some of the radioactivity (30 to 40 percent) did not migrate with Phelac, probably because of rearrangement products (*10*). As shown by Carbon (*11*), these conditions result in minimal alteration of the tRNA. These conditions are milder than those used by Herve and Chapeville (*8*) for the same conversion.

the question of whether the ribosomal system is able to form successive ester links in the polymer chain. In the present work polyuridylic acid [poly(U)] is used as a synthetic messenger and the deaminated phenylalanyl-tRNA (Phelac-tRNA) (Fig. 1) is used in place of phenylalanyl-tRNA. Here we show that the *Escherichia coli* ribosome is able to incorporate the α-hydroxy acid into a polymer chain to form polyesters.

When Phelac-tRNA is incubated in a system for synthesizing protein in vitro, with poly(U) as a synthetic messenger RNA, it is incorporated into an acid-insoluble product (Fig. 2a). The incorporation is assayed by precipitation with cold 10 percent trichloroacetic acid (TCA) after treatment with pancreatic ribonuclease and ethylenediaminetetraacetate (EDTA) under conditions which solubilize both ribosome bound and unbound tRNA. The completeness of tRNA hydrolysis was demonstrated in other control experiments. Incorporation can also be observed by simply precipitating with cold 10 percent TCA and heating the precipitate to 90°C for 15 minutes. The tRNA is also hydrolyzed under these conditions, and the result is the same as that indicated in Fig. 2. The time course of incorporation is similar to that observed for phenylalanine under the same conditions, but the amount of incorporation is less (Table 1). Similarly, there is no incorporation in the absence of ribosomes, supernatant (S-100) or without polyuridylic acid.

The appearance of acid-insoluble material can be interpreted as a polymerization by analogy with the properties of polypeptide synthesizing systems. In the polymerization of phenylalanine with poly(U), treatment with TCA precipitates polymers containing four or more residues (*4*). The polymeric nature of the present product is also shown by treatment with α-chymotrypsin, an enzyme known to hydrolyze both ester and amide linkages. The incorporation product is made acid-soluble by this treatment.

Further characteristics of the incorporation are shown in Table 1. Guanosine triphosphate (GTP) is required, and the GTP requirement cannot be satisfied by the β-γ methylene analog of GTP (designated GMP-PCP). Furthermore, the analog inhibits the incorporation in the presence of an endogenous low concentration of GTP (experiment 1, Table 1). The analog GMP-PCP is active in the enzymatic

Table 1. Incorporation of phenyllactic acid into acid-insoluble precipitate. The complete system in experiment 1 was as described in Fig. 2. The complete system in experiment 2 was identical, but without phosphoenolpyruvate (PEP). Samples (0.05 ml) with and without poly(U) were incubated for 45 minutes at 30°C, then assayed by the ribonuclease–cold TCA procedure described in Fig. 2. For experiment 3, the complete system was as described in Fig. 2, with the use of either (A) phenyllactyl-tRNA or (B) phenylalanyl-tRNA. Samples (0.05 ml) were incubated with and without poly(U) for 15 minutes at 30°C, then assayed by the ribonuclease–cold TCA procedure (Fig. 2). The percentage of inhibition was calculated after subtracting the incorporation in the absence of poly(U).

Reaction	Phelac incorporated		
	+ Poly(U) (pmole)	− Poly(U) (pmole)	Inhibition (%)
Experiment 1			
Complete (including PEP and 0.5 m*M* GTP)	0.57	0.05	
Minus GTP	0.44	0.05	
Minus GTP plus 0.5 m*M* GMP-PCP	0.19	0.05	
Minus GTP plus 1.2 m*M* GMP-PCP	0.13	0.05	
Experiment 2			
Complete (minus PEP but with 0.5 m*M* GTP)	0.64	0.11	
Minus GTP	0.10	0.08	
Minus GTP plus 0.5 m*M* GMP-PCP	0.10	0.08	
Experiment 3			
A. Phelac incorporation			
Complete system	0.50	0.07	
+ Chloramphenicol (2 m*M*)	0.35	0.08	37
+ Gougerotin (2 m*M*)	0.13	0.08	88
B. Phe incorporation			
Complete system	5.10	0.36	
+ Chloramphenicol (2 m*M*)	2.38	0.24	55
+ Gougerotin (2 m*M*)	1.22	0.32	81

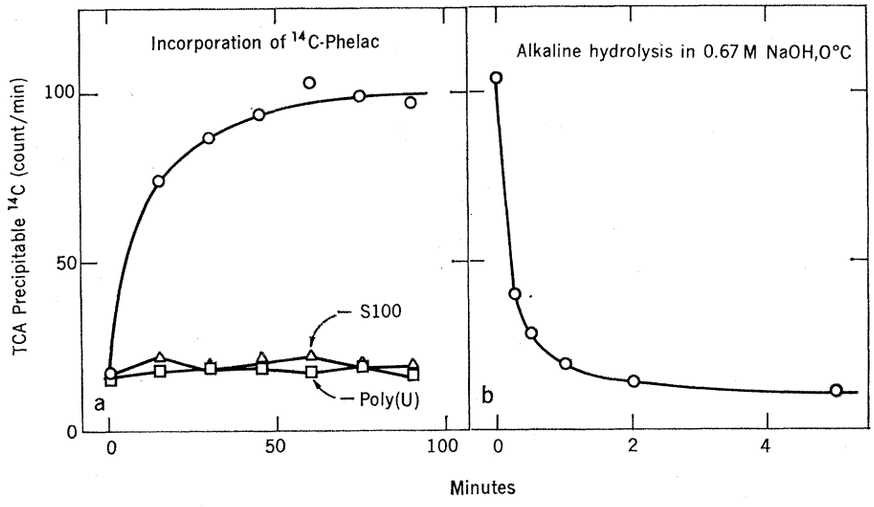

Fig. 2. Incorporation of phenyllactic acid into insoluble precipitate. (a) Kinetics of the incorporation. The complete incorporation system contained 0.11*M* tris-HCl (*p*H 7.4); 0.096*M* NH_4Cl; 0.016*M* magnesium acetate; 0.5 m*M* GTP; 4 m*M* phosphoenolpyruvate (PEP); 0.89 mg of *E. coli* S-100 protein (*1*); ribosomes (2.6 A_{260} unit/ml) (*1*); [^{14}C]Phelac-tRNA (10 A_{260} unit/ml) (28 pmole of Phelac per A_{260} unit, at 459 mc/mmole); and poly(U) (24 μg/ml). Similar portions were incubated without poly(U) or without S-100. The incorporation was initiated by transfer from 0° to 30°C. After various periods of incubation at 30°C, 0.5-ml portions were transferred to tubes containing 0.025 ml of 0.1*M* EDTA with 12.5 μg of ribonuclease. The ribonuclease digestion was carried out for 15 minutes at 30°C, then the tubes were chilled and 0.05 ml of bovine serum albumin (BSA, a solution of 2.5 mg/ml) and 2 ml of cold, 10 percent TCA containing 0.5 percent (weight to volume) casamino acids were added. Acid-precipitable material was collected on Millipore filters and counted by a gas flow counter. (b) Alkaline digestion of acid-precipitable product. Incorporation of Phelac-tRNA was carried out as described above, in a volume of 0.25 ml. After incubation for 45 minutes at 30°C, 0.05 ml of 0.2*M* EDTA and 0.05 ml of ribonuclease (1 mg/ml) were added, followed by a second incubation at 30°C for 15 minutes. Carrier BSA (2 μl, 30 percent weight to volume) was added, and the sample was divided into 0.05-ml portions which were chilled to 0°C. Cold (0°C) 1*N* NaOH (0.1 ml) was added to all but one portion (the zero time point). After the appropriate time at 0°C, 2 ml of cold 10 percent TCA containing 0.5 percent casamino acids was added, the samples were filtered, and the radioactivity was determined by counting.

binding of aminoacyl-tRNA to ribosomes (*5*) but does not support amino acid polymerization. The incorporation of phenyllactic acid thus requires the presence of all of the components necessary for polyphenylalanine formation in similar systems (*6*). This suggests that the observed incorporation is functionally related to polypeptide synthesis.

The relation of the Phelac incorporation to protein synthesis is further indicated by the effect of chloramphenicol and gougerotin (Table 1). These inhibit protein synthesis by blocking the peptidyl transferase (*7*). The degree of inhibition of Phelac incorporation is comparable to the inhibition of polyphenylalanine formation under similar conditions. Variation of the Mg^{2+} concentration in the reaction mixture shows that Phelac and phenylalanine (Phe) incorporations are optimal in the range 12 to 18 m*M*. Thus it is likely that the same ribosomal mechanism is active in the polymerization of both α-amino and α-hydroxy residues.

Despite the great similarity in the incorporation, the products of the reaction with phenylalanine or Phelac differ considerably. The precipitate of the Phelac incorporation was examined after hydrolysis in 6*N* HCl (18 hours, 110°C). With the use of the chromatographic system described by Herve and Chapeville (*8*), the hydrolysis product cochromatographed with phenyllactic acid (R_F = 0.84) and there was no detectable trace of radioactive phenylalanine (R_F = 0.50). Polypeptides are known to be stable in alkali, while esters are labile. Figure 2b shows the effect of alkaline treatment at 0°C on the product of Phelac incorporation. There is a rapid disappearance of the TCA-precipitable material. When phenylalanyl-tRNA is used in the system, there is no change in the yield of precipitable material, even after treatment in 1*N* NaOH for 2 hours at 40°C.

Table 2. Phenyllactic acid incorporation in the presence of phenlyalanine. Incorporation was carried out in the standard system described in Fig. 1. Each sample contained 3 μl of a mixture of solutions of [^{12}C]phenyllanyl-tRNA (A_{260} = 200) and [^{14}C]phenyllactyl-tRNA (A_{260} = 200, 28 pmole of Phelac per A_{260} unit) in the proportion shown. Duplicate samples were incubated with and without poly(U). After 60 minutes incubation at 30°C, the samples were treated with EDTA and ribonuclease as described in Fig. 1, and carrier bovine serum albumin (0.05 ml, 2.5 mg/ml) was added. Alkali lability was measured by treating with 0.5 ml of 1*N* NaOH for 15 minutes at 40°C. All samples were precipitated with 2 ml of cold 10 percent TCA containing 0.5 percent (weight to volume) casamino acids. The values of incorporation without poly(U) were subtracted to obtain the values listed.

Input ratio [^{14}C]Phelac: [^{12}C]Phe	Phelac		
	Precipitated (pmole)	Alkali labile (%)	Incorporated (% of input)
1 : 0	0.56	98	6.7
1 : 1	0.25	35	5.9
1 : 2	0.19	33	6.9
1 : 4	0.104	17	6.1

These experiments indicate that an alkali-labile, acid-precipitable product is formed when [^{14}C]Phelac-tRNA is used in the incorporation system. Phenylalanine is not present in the system initially, because the supernatant is dialyzed. However, the supernatant is unfractionated, and it may contain proteolytic enzymes that liberate phenylalanine during the incubation. Thus, to demonstrate the presence of consecutive residues of Phelac in the polymer, it is necessary to analyze the products of alkaline digestion and to show that free phenyllactic acid is produced. An analysis which also measured the amount of phenylalanine incorporation was carried out. This analysis used the known lability of ester linkages and the stability of peptide linkages. After alkali treatment, the products were analyzed by paper electrophoresis at *p*H 5.3 and by silica gel chromatography in two solvent systems [*n*-butanol, acetic acid, H_2O (78 : 5 : 17) and isoamyl alcohol, acetone, acetic acid, H_2O (25 : 25 : 1 : 1)]. The major component in the alkali-treated material coincides with the phenyllactic acid standard in all three systems (Fig. 3). In addition, a smaller radioactive peak migrates ahead of the Phelac standard, together with the dipeptide standard phenyllactyl-phenylalanine (Phelac-Phe). If phenylalanine were incorporated in the system, it would be joined in phenyllactic acid by a peptide bond through its α-amino group, and this bond would be stable to alkaline hydrolysis. The radioactivity in the Phelac-Phe peak is due to [^{14}]CPhelac. Analysis of this experiment suggests that 70 to 80 percent of the residues in the precipitated polymer are phenyllactic acid residues, and the remaining residues are probably phenylalanine. Thus, the acid-insoluble polymer is largely a polyester with some amide linkages incorporated as well.

Herve and Chapeville (*8*) have reported that Phelac-tRNA is incorporated in the NH_2-terminal position of polyphenylalanine in a system primed with poly(U). We have been able to confirm these results by adding unlabeled phenylalanine to our incorporation mixture. In the NH_2-terminal position of a polypeptide [^{14}C]Phelac would not be sensitive to alkaline hydrolysis. In the experiment described in Table 2, ^{14}C-labeled Phelac-tRNA and unlabeled Phe-tRNA were mixed in various proportions, and the products were examined for alkaline lability. The total amount of tRNA is the same in each incubation. The fraction of the input Phelac incorporated is approximately constant, but as more Phe is put into the reaction mixture the fraction of the alkali-labile [^{14}C]-Phelac decreases. This alkali-resistant

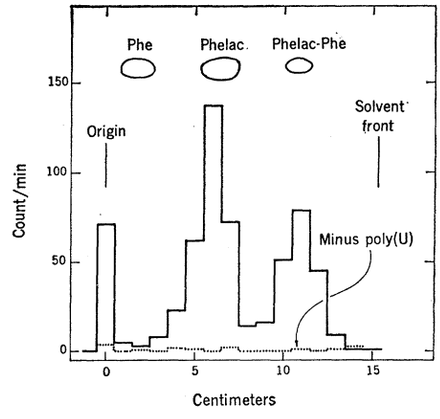

Fig. 3. Characterization of the alkaline-digested product. Incorporation of Phelac-tRNA was carried out as described in Fig. 2. Portions (1 ml) were incubated with and without poly(U), and then treated in parallel. After a 60-minute incubation at 30°C, 0.2 ml of 0.2*M* EDTA and 0.2 ml of ribonuclease (1 mg/ml) were added, and the samples were incubated 15 minutes at 30°C. Cold 10 percent TCA (2 ml) was added, and the precipitate was collected by centrifugation. The precipitates were then washed twice with 5 ml of cold 5 percent TCA by vigorous mixing on a Vortex mixer, followed by centrifugation. The TCA was removed by lyophilization, and the residue was taken up in 0.1 ml of 1*N* KOH and incubated for 45 minutes at 40°C. The samples were neutralized with 1*N* $HClO_4$, and after chilling to 0°C, the precipitated $KClO_4$ was removed by centrifugation. Carrier phenyllactic acid and phenylalanine (20 μg each) were added. A portion of each of these samples was then subjected to ascending chromatography on Eastman silica gel G thin layers in a system containing isoamyl alcohol, acetone, acetic acid, and water (25 : 25 : 1 : 1). Silica gel plates were cut into 1-cm strips, and the radioactivity was counted in a liquid-scintillation spectrometer.

incorporation is similar to that described by Herve and Chapeville and is clearly distinguishable from the alkali-labile incorporation described above. Thus, an incorporation system with substantial amounts of phenylalanine would show mainly NH_2-terminal incorporation of Phelac, instead of polyester formation. It is likely that this explains the difference between our results and the work reported by Herve and Chapeville.

We have attempted to measure the chain length of the polyester by a method closely related to that of Herve and Chapeville (*8*). In their experiments, ^{14}C-labeled phenyllactate is attached to ^{32}P-labeled tRNA. In the absence of GTP, the ratio of ^{14}C to ^{32}P is measured to assay the ribosomal bound Phelac-tRNA in the absence of polymerization. In the presence of GTP, polymerization ensues, and the ratio increases when more than one ^{14}C residue is bound to the tRNA in the nascent polymer. If nonspecific binding of Phelac-tRNA occurs even in the presence of GTP, then this measurement gives a lower limit for the actual polymer length. The results of Herve and Chapeville showed no increase in the ratio in the presence of GTP. This was interpreted to indicate the presence of only one Phelac residue per polypeptide. We have repeated this measurement (*9*) and find that GTP induces an increase in [^{14}C]Phelac bound of 14.7-fold relative to that bound in the presence of the analog GMP-PCP. This shows that polymerization has occurred, but in view of the possibility of nonspecific binding of tRNA to the ribosome, this number should not be interpreted quantitatively.

In most of these experiments the incorporation of ^{14}C radioactivity into insoluble precipitate was less than 10 percent of the input radioactivity. It is likely that the incorporation is limited because of racemization and rearrangement of the amino acid which occurs during nitrous acid deamination of phenylalanyl-tRNA. In analogous experiments with nitrous acid deamination of puromycin (*1*), it was found that the majority of the deaminated material had undergone rearrangement, leaving only a small proportion with the original active configuration. The mechanism of these rearrangements has been studied in detail in simpler molecules (*10*).

In our work with puromycin analogs, we showed that the ribosomal peptidyl transferase was able to form ester bonds as well as amide bonds (*1*). In an extension of this work we used the natural messenger RNA found in bacteriophage R17 and showed that the incorporating system was able to insert α-hydroxy acids into unique positions in a coat protein fragment (*3*). Our results here demonstrate that the ribosome can form consecutive ester bonds and produce a polyester when it has as a substrate α-hydroxyacyl tRNA. Analysis of the product suggests some incorporation of phenylalanine into the polyesters so that the product might be more properly described as a mixed polyester-amide. However, it is likely that incorporation of phenylalanine in the system could be eliminated entirely by fractionating the incorporating system and eliminating the peptidases which generate phenylalanine during the course of the incubation.

Demonstration of the fact that the ribosomal peptidyl transferase can utilize an α-hydroxyl residue as well as an α-amino residue in the synthetic reaction may have some bearing on the possibility that peptidyl transferase catalyzes the hydrolytic reaction in chain termination (*1*, *3*). In addition it may be related to the fact that peptidases which cleave peptide bonds also have the ability to cleave ester bonds. The specificity for both the synthetic and the hydrolytic reactions is similar. This similarity may be of value in understanding the mode of action of peptidyl transferase, an enzyme that occupies a central role in molecular biology.

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Sex Difference in the Number of Sympathetic Neurons in the Spinal Cord of the Cat

Abstract. *Counts of preganglionic sympathetic neurons in the spinal cords of four male and four female cats were obtained. The number of these neurons in males was significantly greater than that in females. These data suggest that the numbers of neurons in the mammalian central nervous system are different in the two sexes.*

It is well established that the mean weight of the brain of men is greater than that of women and that brain weight in the two sexes is poorly correlated with body weight and length (*1*). A similar sex difference has been demonstrated for the weight of the human cerebellum (*2*) and for the volume of the spinal cord in cats (*3*). At least four factors must be considered in attempts to account for these differences in weight and volume: (i) a difference in the number of neurons (*4*), (ii) a difference in their size, (iii) a difference in the number of glial cells (*5*), and (iv) a difference in the development of dendrites, which is usually expressed as the ratio of neuropil to perikaryon (*6*).

We now report data demonstrating that the number of sympathetic preganglionic neurons in the spinal cord of the cat is greater in the male than in the female. In the course of an investigation of descending autonomic connections between the medulla and the spinal cord it became necessary to study the numerical distribution of neurons in the thoracolumbar intermediolateral nucleus (ILN) of the spinal cord, and the results presented here are part of a larger study to be published elsewhere (*7*).

Eight adult cats (2.2 to 3.0 kg) were anesthetized and perfused with 0.9 percent saline and then with 10 percent formol in saline. The spinal cords were removed and fixed in 10 percent for-