Effects of Amino Acids, s-RNA, and Ethanol on Coding Ambiguity with Polyuridylic Acid*

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ABSTRACT: The effect of free amino acids and soluble ribonucleic acid (s-RNA) concentration on the specificity of amino acid incorporation by polyuridylic acid (poly-U) in an *Escherichia coli* cell-free system has been studied. Addition of excess s-RNA stimulates phenylalanine incorporation up to 20-fold, strongly inhibits isoleucine incorporation, and has little or no effect on leucine incorporation. Phenylalanine stimulates leucine incorporation 50–100%, and in the presence of excess s-RNA and ethanol the stimulation ranges from 200 to 500%. Leucine inhibits isoleucine incorporation in the presence or absence of supplementary s-RNA, whereas other amino acids such as phenylalanine, valine, tyrosine, and serine show little or no effect. In the presence of ethanol and supplementary s-RNA, inhibition of isoleucine incorporation by leucine is more pronounced, and under these conditions phenylalanine also inhibits isoleucine incorporation. The results were explained on the basis that copeptides are synthesized in the presence of poly-U under conditions of ambiguous coding and that competition exists among different aminoacyl s-RNA's for a common binding site on the poly-U-ribosome complex. An explanation which accounts in part for the poly-U ambiguity was suggested and involves tautomerism of uracil in messenger ribonucleic acid or other purine and pyrimidine bases in s-RNA.

The importance of environmental factors on the incorporation of various amino acids in cell-free preparations from microorganisms has been noted by a number of investigators. Organic solvents (So and Davie, 1964; Sarin and Zamecnik, 1965), magnesium ion and temperature (Szer and Ochoa, 1964; Friedman and Weinstein, 1964), ammonium ion and polynucleotide concentration (So and Davie, 1964; So *et al.*, 1964), polynucleotide size (Sager, 1963), and streptomycin (Davies *et al.*, 1964) all influence the specificity of amino acid incorporation in response to a particular polynucleotide. Grunberg-Manago and Dondon (1965) have also provided evidence for the importance of s-RNA concentration and pH on amino acid ambiguity. Effects of s-RNA and streptomycin on the binding of aminoacyl-s-RNA to the poly-U-ribosome complex were also reported recently by Pestka *et al.* (1965).

In extending our earlier studies, the effects of free amino acid and s-RNA were examined and found to have a marked influence on poly-U-dependent incorporation of phenylalanine, leucine, and isoleucine. Many of these effects were potentiated by ethanol. A tautomeric shift in the uracil bases of poly-U or other bases in s-RNA was suggested to account in part for the amino acid ambiguity with poly-U. The current theory of amino acid coding is discussed in terms of these data.

Materials

Polyuridylic acid (poly-U) was purchased from Miles Chemical Co., Elkhart, Ind. According to the manufacturer, its sedimentation coefficient ranged from 4 to 7. s-RNA was prepared from *Escherichia coli* by the method of Holley *et al.* (1961) and freed of residual amino acids by the procedure of Nathans and Lipmann (1961). L-[U-^{14}C]Phenylalanine, L-[U-^{14}C]leucine, and L-[U-^{14}C]isoleucine were purchased from New England Nuclear Corp., Boston, Mass. Crystalline ATP[1] (disodium salt) and GTP were purchased from Pabst Laboratories, Milwaukee, Wis. Phosphoenolpyruvate (trisodium salt), pyruvic kinase, and L-[^{12}C]amino acids (A grade) were purchased from California Corp. for Biochemical Research, Los Angeles, Calif. Column chromatography of L-[^{12}C]leucine by the procedure of Spackman *et al.* (1958) indicated an isoleucine contamination of less than 0.5%. *E. coli* $K_{12}F^-$ was a gift of Mr. Don Brenner. All other chemicals employed were commercially available reagent grade.

Methods

Most of the methods employed in these experiments were reported previously (So and Davie, 1964; So *et al.*, 1964). *E. coli* extracts were prepared essentially by the method of Nirenberg and Matthaei (1961). An S-30 fraction was used throughout these experiments.

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[1] Abbreviations used in this work: ATP, GTP, the 5′-triphosphates of adenosine and guanosine, respectively.

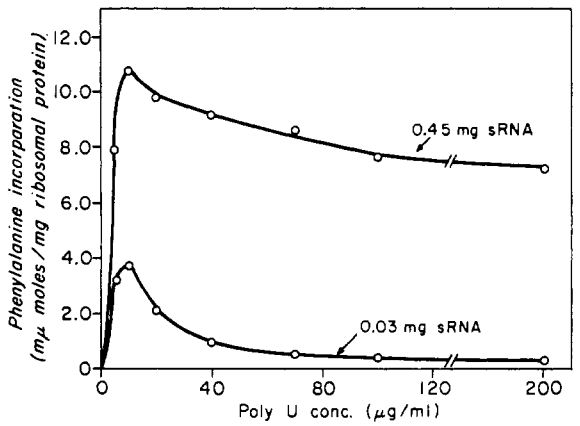

FIGURE 1: The effect of poly-U concentration on phenylalanine incorporation in the presence of 0.03 mg/ml s-RNA and 0.45 mg/ml s-RNA. In addition to the standard reaction mixture, each tube contained 5×10^{-5} M L-[^{14}C]phenylalanine, 1 mg of ribosomal protein, 2.5 mg of supernatant protein, and either 0.03 mg/ml or 0.45 mg/ml of s-RNA. Poly-U concentrations were as shown in the graph.

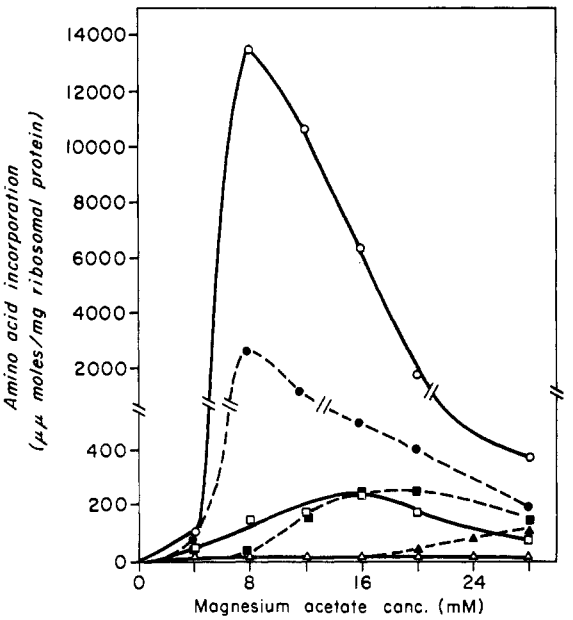

FIGURE 2: The effect of magnesium ion concentration on poly-U-dependent amino acid incorporation at 0.03 mg/ml s-RNA (dashed lines) and 0.45 mg/ml s-RNA (solid lines). In addition to the standard reaction mixture each tube contained 1 mg of ribosomal protein, 2.5 mg of supernatant protein, either 0.030 mg or 0.45 mg of s-RNA, and 5×10^{-5} M L-[^{14}C]amino acid (phenylalanine, O—O; leucine, □—□; isoleucine, △—△). The concentration of poly-U was 100 μg/ml.

The standard incubation mixture contained 0.10 M Tris buffer (pH 7.8), 0.009 M magnesium acetate, 0.006 M mercaptoethanol, 0.06 M ammonium chloride, 0.005 M phosphoenolpyruvate, 0.001 M ATP, 5×10^{-5} M GTP, and 0.035 mg of pyruvic kinase in a final volume of 1 ml. Tubes were routinely incubated for 30 minutes at 25°. Radioactivity was counted by the method of So and Davie (1963) using a Packard scintillation spectrometer Model 314EX. All experimental values reported have been corrected for the background count of the instrument (50–60 cpm) and incorporation in the absence of added synthetic poly-U which generally did not exceed 50 cpm.

Results

Effect of s-RNA on Poly-U-dependent Amino Acid Incorporation. In preliminary experiments, a marked reduction in the incorporation of isoleucine and leucine relative to phenylalanine was observed by increasing concentration of crude *E. coli* supernatant. In these experiments with low ribosomal protein concentration (0.25 mg/ml), the supernatant protein was increased from 0.8 to 3.4 mg/ml. Further studies showed that the supernatant effect was primarily due to its content of s-RNA. The effect of supplementary s-RNA on phenylalanine incorporation with increasing poly-U concentration is shown in Figure 1. The control contained 0.03 mg/ml s-RNA and the supplemented tubes contained 0.45 mg/ml of s-RNA. The added s-RNA contained little or no bound amino acids. It can be seen that there is a marked stimulation of phenylalanine incorporation by s-RNA, and at high poly-U concentrations (100 μg/ml) this stimulation reaches 20- to 30-fold. In the presence of excess s-RNA, little inhibition of phenylalanine incorporation was noted at high poly-U concentrations. In contrast, an inhibition was generally seen at high concentrations of poly-U in the absence of excess s-RNA (Jones *et al.*, 1964; So and Davie, 1964).

In previous studies we have reported the effect of magnesium ion concentrations on the incorporation of phenylalanine, leucine, and isoleucine in the presence of poly-U (So *et al.*, 1964). Figure 2 shows similar studies with 0.03 mg/ml s-RNA and 0.45 mg/ml s-RNA. Phenylalanine incorporation is again stimulated by high levels of s-RNA. In contrast, the addition of excess s-RNA has little or no effect on leucine incorporation and inhibits isoleucine incorporation about 80%.

The inhibition of isoleucine incorporation by increasing concentrations of s-RNA is shown in Table I. In the absence of ethanol, the inhibition of isoleucine incorporation is proportional to increasing concentrations of s-RNA, and this inhibition approached 80% with 0.67 mg/ml of s-RNA. However, in the presence of ethanol, s-RNA concentrations above 0.19 mg/ml did not result in any further inhibition. It should be noted that the incorporation of isoleucine in the presence of ethanol was about four times the incorporation found in the absence of ethanol (1573 *vs.* 360 μμmoles at 0.02 mg/ml s-RNA). In these experiments, the optimal magnesium ion concentration either in the presence or absence of ethanol was employed. In the absence of

TABLE I: Effect of s-RNA Concentration on Isoleucine Incorporation in the Presence and Absence of Ethanol.[a]

Final Concn. s-RNA (mg/ml)	Isoleucine Incorporation ($\mu\mu$moles/mg ribosomal protein) Minus Ethanol	Plus 1.6 M Ethanol
0.02	360	1573
0.18	160	966
0.34	109	926
0.50	93	933
0.66	73	980

[a] Reaction mixtures were the same as the standard incubation mixture described under Methods except that (1) magnesium ion concentration was 0.024 M in the absence of ethanol and 0.009 M in the presence of ethanol; and (2) 1×10^{-5} M L-[^{14}C]isoleucine, 0.6 mg of ribosomal protein, and 1.3 mg of supernatant protein were added. Final concentrations of s-RNA were as indicated in the table. The concentration of poly-U was 100 μg/ml.

alcohol, the optimum was 0.024 M and in its presence, 0.009 M.

Effects of Free Amino Acids on Amino Acid Incorporation by Poly-U. Poly-U-dependent incorporation of phenylalanine, leucine, and isoleucine was also found to be influenced by the addition of free amino acids. The effects of leucine, isoleucine, serine, tyrosine, and valine on phenylalanine incorporation in the presence of ethanol are shown in Table II. At a poly-U concentration of 100 μg/ml, leucine (5×10^{-5} M) inhibits phenylalanine incorporation about 30% at low or high levels of s-RNA, while other amino acids had little or no effect. Similar results were observed in the absence of ethanol either with or without supplementary s-RNA.

The effect of phenylalanine and excess s-RNA on the poly-U-dependent incorporation of leucine in the presence and absence of 0.64 M ethanol is shown in Table III. In the absence of ethanol, phenylalanine stimulates the incorporation of leucine about 50%, whereas excess s-RNA has no effect. In the presence of both phenylalanine and excess s-RNA, the stimulation of leucine incorporation was greater than 200%. A 500% stimulation of leucine incorporation was observed in the presence of phenylalanine, excess s-RNA, and 0.64 M ethanol. The effects of phenylalanine and excess s-RNA were also dependent on the concentration of magnesium ion. Maximal stimulation of leucine incorporation by phenylalanine occurred at 0.016 M magnesium. The addition of isoleucine, serine, valine, and tyrosine had no detectable effect on leucine incorporation in the presence or absence of excess s-RNA. Similarly, these amino acids were without effect on leucine incorporation either in the presence or absence of ethanol.

Leucine, serine, phenylalanine, valine, and tyrosine were also studied for their effect on isoleucine incorporation in the presence of 1.6 M ethanol (Table IV). With 0.03 mg/ml s-RNA, leucine inhibits isoleucine incorporation about 75%, while a very small stimulation is occasionally observed by the addition of other amino

TABLE II: Effects of Various Amino Acids on Phenylalanine Incorporation in the Presence of Ethanol.[a]

Amino Acid Added (5×10^{-5} M)	Phenylalanine Incorporation ($\mu\mu$moles/mg ribosomal protein) Low s-RNA	High s-RNA
Control	4600	10,700
Leucine	2900	7,400
Isoleucine	4600	10,400
Serine	5000	10,900
Tyrosine	4500	11,200
Valine	4800	10,400

[a] In addition to standard incubation mixture each tube contained 0.6 mg ribosomal protein, 1.3 mg supernatant protein, and 1×10^{-5} M L-[^{14}C]phenylalanine. The experiments also contained 100 μg/ml of poly-U and 0.8 M ethanol. The concentration of s-RNA for low s-RNA was 20 μg/ml and for high s-RNA was 0.18 mg/ml.

TABLE III: Effect of Phenylalanine and s-RNA on Leucine Incorporation in the Presence and Absence of Ethanol.[a]

Tube	Phenylalanine	s-RNA (mg/ml)	Leucine Incorporation ($\mu\mu$moles/mg ribosomal protein) Minus Ethanol	Plus Ethanol
1	−	0.03	244	379
2	+	0.03	331	1212
3	−	0.19	246	433
4	+	0.19	564	1793

[a] Incubation mixtures were the same as the standard incubation mixture described under Methods except that (1) magnesium ion concentration was 0.009 M in the presence of 0.64 M ethanol and 0.016 M in the absence of ethanol; and (2) 1×10^{-5} M L-[^{14}C]leucine, 6.0 mg of ribosomal protein, and 1.3 mg of supernatant protein were added. Each reaction mixture also contained 100 μg/ml of poly-U. Tubes 2 and 4 contained 5×10^{-5} M phenylalanine.

TABLE IV: Effect of Amino Acids on Isoleucine Incorporation in the Presence of Ethanol.[a]

Amino Acid Added (1×10^{-5} M)	Isoleucine Incorporation ($\mu\mu$moles/mg ribosomal protein) Low s-RNA (0.02 mg/ml)	High s-RNA (0.18 mg/ml)
	1147	913
Leucine	265	241
Phenylalanine	1273	660
Tyrosine	1333	913
Serine	1207	847
Valine	1147	927

[a] In addition to the standard incubation mixture each tube contained 0.6 mg ribosomal protein, 1.3 mg supernatant protein, and 1×10^{-5} M L-[^{14}C]isoleucine. The concentration of poly-U was 100 μg/ml and that of ethanol 1.6 M.

acids. In the presence of 0.45 mg/ml s-RNA, a similar degree of inhibition was noted with leucine and, in addition, phenylalanine consistently inhibits isoleucine incorporation about 25%. Similar but less pronounced effects were observed in the absence of ethanol where the incorporation of isoleucine was inhibited about 20–30% by leucine. This inhibition was further increased to 70–80% by the addition of 0.15 mg/ml of s-RNA. Addition of excess s-RNA alone inhibited isoleucine incorporation about 40% under these conditions.

It was then of interest to test whether the effects of free amino acids and ethanol on the over-all process of protein biosynthesis were related to the various aminoacyl-s-RNA synthetases. It was found that the esterification of phenylalanine, leucine, or isoleucine by s-RNA either in the presence or absence of ethanol was not affected by other amino acids up to a concentration of 5×10^{-5} M, the concentration where optimal effects of amino acids on poly-U-dependent amino acid incorporation was observed. Thus the effects of amino acids appear to be related to the steps in protein synthesis where amino acids are transferred from aminoacyl-s-RNA to the m-RNA-ribosome complex and polymerized.

Discussion

The present experiments clearly show that amino acid incorporation in response to specific polynucleotides is influenced by the presence of additional amino acids and the concentration of s-RNA in the incubation mixture. These effects were more pronounced in the presence of ethanol. In previous studies dealing with amino acid ambiguity, the effects of concentrations of s-RNA and free amino acids generally were not taken into consideration. Various investigators using poly-U as an m-RNA either included each of the nineteen L-[^{12}C]amino acids along with L-[^{14}C]amino acid in their reaction mixture (Davies *et al.*, 1964; Friedman and Weinstein, 1964), or included only the radioactive amino acid being studied, and thus had only low levels of endogenous amino acids present (So and Davie, 1964; Szer and Ochoa, 1964). Similar variations exist with the concentration of s-RNA in that some investigators (Szer and Ochoa, 1964) fortified the reaction mixture with additional s-RNA while others (So and Davie, 1964; So *et al.*, 1964; Friedman and Weinstein, 1964; Davies *et al.*, 1964) relied on the endogenous s-RNA already present in the crude extract.

The incorporation of phenylalanine, leucine, and isoleucine in the presence of poly-U responded differently to the addition of supplemental s-RNA and various amino acids. Supplementary s-RNA stimulated the incorporaton of phenylalanine, inhibited isoleucine incorporation, but had little or no effect on leucine incorporation. Similar results were reported recently by Grunberg-Manago and Dondon (1965).

In the absence of ethanol, free amino acids had little or no effect on phenylalanine incorporation. However, in the presence of ethanol, leucine consistently reduced phenylalanine incorporation.

The incorporation of isoleucine was inhibited by the addition of either leucine or supplementary s-RNA, and these effects were potentiated by the presence of ethanol. Control experiments have shown that the binding of isoleucine to s-RNA either in the presence or absence of ethanol is not affected by other amino acids in this range of concentration. Thus it appears likely that leucyl-s-RNA is competing with isoleucyl-s-RNA for a common binding site on the poly-U-ribosome complex. It also appears that the binding of leucyl-s-RNA to the poly-U-ribosome complex is favored over the binding of isoleucyl-s-RNA since the presence of isoleucine is without effect on leucine incorporation.

It has been suggested that the amino acid ambiguity found with poly-U may be explained by base pairing involving only two rather than three bases in the triplet code (Grunberg-Manago and Michelson, 1964; Friedman and Weinstein, 1964). This conclusion was based upon the fact that the code words for leucine, isoleucine, tyrosine, and serine all contain two U's and all these amino acids are incorporated to some extent in the presence of poly-U. If pairing of only two of the three bases were sufficient to code for a single amino acid, then a competition should exist among all these amino acids. In the present experiments, the competition was quite specific in that only leucine inhibited phenylalanine and isoleucine incorporation. These experiments suggest that all the bases in the triplet code are functional; i.e., all the bases of the triplet are read by s-RNA during ambiguous coding.

The incorporation of leucine at its optimal magnesium ion concentration was stimulated by phenylalanine. This stimulation was further increased by the addition of ethanol. The stimulation of leucine incorporation

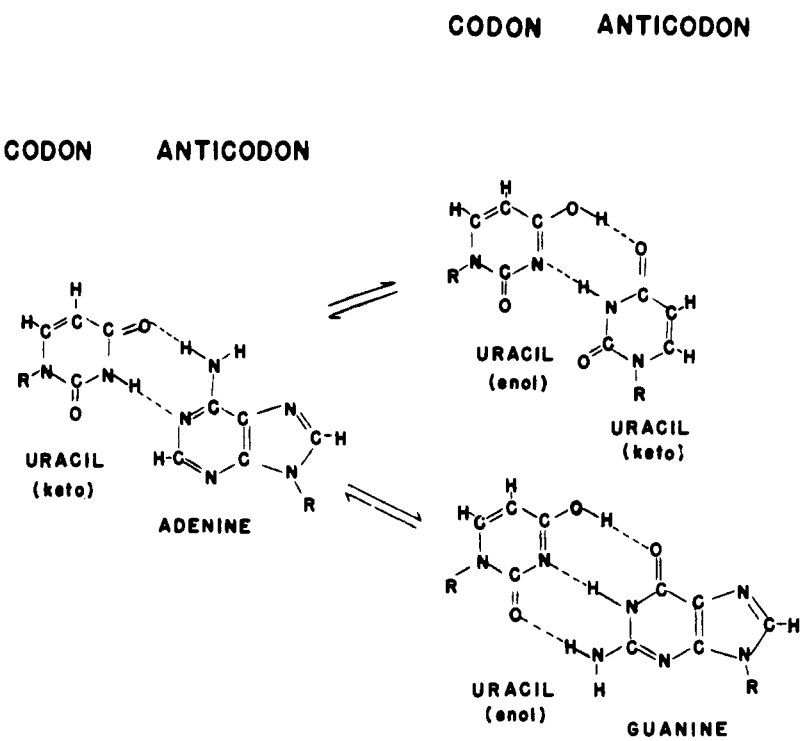

FIGURE 3: Possible base pairings for keto and enol forms of uracil.

suggests the copeptides of leucine and phenylalanine are being synthesized. It is likely that the number of coding sites for leucine in poly-U is small compared with those which code for phenylalanine. Thus phenylalanine may form bridges between leucine residues. This could lead to an increase in the incorporation of leucine into a product precipitable by acid. These results differ from those of Jones and Nirenberg (1962) and Szer and Ochoa (1964), who reported an inhibition of leucine incorporation by phenylalanine. The reasons for these differences will require further study.

The specificity of coding is presently believed to occur through hydrogen bonding between three bases in m-RNA and their complementary bases in aminoacyl-s-RNA (Crick, 1957, 1964; Hoagland, 1959; Nirenberg and Leder, 1964; Nishimura *et al.*, 1965). Miscoding occurs when the normal interaction of the aminoacyl-s-RNA's with the m-RNA-ribosome complex is modified. This may be owing to a number of reasons such as conformational changes in RNA which might permit unusual base pairings at the coding site. As previously suggested (So and Davie, 1964), amino acid ambiguity found with poly-U in the presence of organic solvents may be explained in part by a tautomeric shift of bases in poly-U or s-RNA, resulting in the pairing of uracil with bases other than adenine. These tautomeric shifts have been previously suggested as a mechanism for mutations during DNA replication (Watson and Crick, 1953; Freese, 1959; Stahl, 1964). This change in base pairing could explain the finding that one of the uracil bases in the UUU triplet could function either as C and code for leucine and serine, or function as A and code for isoleucine and tyrosine. Such a tautomeric shift could explain the competition of leucine for the incorporation of isoleucine, but the competition of leucine for phenylalanine is not obvious.

A tautomeric shift of uracil and the possible base pairings of the two tautomers is illustrated in Figure 3. The important feature is the conversion of a hydrogen bond acceptor (keto group) to a hydrogen bond donor (enol group) and the simultaneous conversion of N-3 from a hydrogen donor to a hydrogen acceptor. The classical Watson-Crick base pairing is shown on the left-hand side for uracil in its keto form with adenine. If enolizaton occurs, either at C-2 or C-4 of uracil, this base will no longer pair with adenine. It will, however, pair with another uracil in the keto form or with guanine. The pairing with guanine probably would be more favorable since three hydrogen bonds would be possible. Furthermore, geometrical spacing also may be important since the latter involves the typical purine-pyrimidine pairing. If enolization occurred in the first uracil of a codon triplet in poly-U, i.e., terminal 5′-phosphate, it then could base pair with the anticodons of GAA or UAA in s-RNA. These are the anticodons for leucine and isoleucine whose codon triplets include CUU and AUU, respectively (Bernfield and Nirenberg, 1965; Trupin *et al.*, 1965). Enolization in the second position of a codon triplet in poly-U would permit base pairing of the anticodons possessing AGA or AUA in s-RNA's. These anticodons would correspond to serine and tyrosine whose codons are UCU and UAU, respectively (Bernfield and Nirenberg, 1965; Trupin

et al., 1965). Enolization in the third position of a codon triplet in poly-U would permit base pairing of the anticodons possessing AAG in s-RNA's. AAG corresponds to one of two anticodons of phenylalanine whose code triplet includes UUU and UUC (Bernfield and Nirenberg, 1965). Thus far, no amino acid assignment for the code word UUA has been made. If enolization occurred in a random distribution in all three positions of the triplet code, then serine and tyrosine should be incorporated to about the same extent as leucine and isoleucine. The reasons for the preferential incorporation of leucine and isoleucine over serine and tyrosine under the present conditions are not known.

A tautomeric shift could also occur in bases in the anticodon of s-RNA. For instance with isoleucyl s-RNA, which has an anticodon of UAA, an enolization in uridine would permit pairing of triplet anticodon bases in isoleucyl-s-RNA with the keto forms of UUU in poly-U. This would again lead to the incorporation of isoleucine with poly-U.

The experiments of Grunberg-Manago and Michelson (1964) with polybromouridylic acid are consistent with the keto-enol proposal. They reported that polybromouridylic acid stimulated the incorporation of phenylalanine, leucine, and isoleucine, and to a lesser extent, serine and tyrosine. The incorporation of leucine, however, was greater than that of phenylalanine. These are the same amino acids which are incorporated by poly-U under our present experimental conditions. Substitution of bromine at C-5 in uracil would favor enolization of the keto group at the C-4 position. This could then lead to the typical poly-U ambiguity. Indeed, these authors pointed out the possible importance of tautomerizaton. They have also suggested, however, a base pairing between uracil and bromouracil involving hydrogen bonds with bromine to account for their findings.

Tautomerization in various codon bases of m-RNA or anticodon bases of s-RNA could also be important for other amino acid ambiguities observed in cell-free systems. These mistakes would be particularly evident if environmental factors potentiate tautomerization, or stabilize enol or imino forms of the purine or pyrimidine bases. For instance, cytosine in the imino form could base pair with the usual forms of adenine or cytosine. Similarly, adenine in the imino form could base pair with cytosine or adenine, and guanine in the enol form could base pair with uracil and guanine. Such combinations could lead to considerable ambiguity in cell-free systems where environmental factors are not closely regulated. Whether amino acid ambiguity caused by factors such as changes in temperature, magnesium ion concentration, or pH, or by the addition of antibiotics such as streptomycin, may be related to tautomerism is not known, since only a few studies have been made on the tautomerization of purine and pyrimidine bases in nucleic acid (Miles, 1961; Gatlin and Davis, 1962). Thus further studies will be required to test the importance of tautomerism as a cause of ambiguous coding in protein biosynthesis.

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O,5′-Thymidylyl-L-serine: A Model for Possible Linkers in DNA*

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ABSTRACT: *O*,5′-Thymidylyl-L-serine, a model for possible linkers in deoxyribonucleic acid (DNA), was prepared by two synthetic methods. This compound was unaffected by a wide variety of exo- and endonucleases or peptidases, as well as various chemical agents, such as hydroxylamine and sodium hyponitrite. However, it was cleaved into serine and thymidylic acid by snake venom phosphodiesterase. These properties should prove useful in attempts to identify such linkers in DNA.

Discussion

The proposal (Bendich and Rosenkranz, 1963) that seryl (or threonyl) oligopeptides (I, Ia, Ib) may serve as linkers or "punctuation" in the genetic code of deoxyribonucleic acids (DNA) prompted the synthesis of model compounds for aid in the identification of such fragments in partial hydrolysates of DNA. The proposal was based, in part, on the isolation of amino acids, including *O*-phosphoserine, from mineral acid hydrolysates of rigorously deproteinized DNA's from mammalian, bacterial, and viral sources and the fragmentation of DNA by hydroxylamine derivatives and alkali (Borenfreund *et al.*, 1961; Bendich and Rosenkranz, 1963; Bendich *et al.*, 1964; and references cited therein). The natural occurrence of amino acids in DNA appears to be widespread (Balis *et al.*, 1964; Champagne *et al.*, 1964; Olenick and Hahn, 1964).

The carboxylic ester linkage in II is analogous to the bond uniting the amino acid or growing peptide chain to the 3′- (or 2′-) hydroxyl of the terminal adenosine in transfer RNA's (Hoagland *et al.*, 1958; Zachau *et al.*, 1958; Hecht *et al.*, 1959; Zachau, 1960; Zachau and Karau, 1960; Nathans *et al.*, 1962; Feldman and Zachau, 1964). The serine nucleotide shown in III has not yet, to our knowledge, been found in nature. Since serine and threonine constituted about one-third of the amino acids isolated from the DNA of bull sperm (Borenfreund *et al.*, 1961), it was suggested that the seryl peptide was a small one, containing perhaps only a few amino acids (Ib).

The identification of fragments similar to II or III in appropriate partial hydrolysates of DNA would constitute persuasive evidence for the existence of such linkages within nucleic acids. To this end, a knowledge of the properties of such possible fragments of known structure would be helpful. Efforts were directed toward the synthesis of nucleotide derivatives similar to III since they would be expected to be more stable than those of type II. For instance, hydroxylamine or mild alkali would be expected to hydrolyse II but not III (Zachau, 1960; Zachau and Karau, 1960; Sokolova *et al.*, 1962). The phenylalanyl ester of thymidine (II, R = benzyl) has been prepared (Sokolova *et al.*, 1963) and found to cleave readily with hydroxylamine.

Results

Two straightforward synthetic routes (Scheme I) to *O*,5′-thymidylyl-L-serine (IV) were attempted, and both led to the desired product. (A) Adaptation of the dicyclohexylcarbodiimide phosphodiester synthesis of Khorana using *N*-carbobenzyloxy-L-serine benzyl ester

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