*DENATURED DNA AS A DIRECT TEMPLATE FOR IN VITRO PROTEIN SYNTHESIS**

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We recently reported[1] that certain preparations of DNA obtained from mammalian cells grown in cell culture were capable of markedly stimulating protein synthesis by an *Escherichia coli* cell-free system. It appeared that single-stranded DNA was acting directly as a template for protein synthesis. A detailed study of the requirements for this effect showed that RNA synthesis is not involved.[1, 2] DNA from other sources was found to be essentially inactive. Clearly it would be of great interest if this effect could be generalized since it could provide a means for direct translation of the genetic code of DNA into amino acid sequences. It will be shown below that streptomycin, neomycin, and kanamycin can greatly enhance the ability of single-stranded DNA from every source tested to stimulate protein synthesis by an *E. coli* cell-free system. The unique properties of DNA prepared from mammalian cells grown in culture result from its exposure to one or more of these antibiotics in the growth medium.[2]

Materials and Methods.—Cell-free protein synthesis was measured with extracts of *E. coli* strain K12 as previously described.[1] The radioactive amino acids used were all obtained from New England Nuclear Corp.

DNA was prepared from various sources by the methods of Berns and Thomas,[3] and Marmur.[4] The various DNA's of different base compositions[5] originated from the following organisms: *Staphylococcus aureus*, 35 per cent GC; *Bacillus subtilis*, 42 per cent GC; *E. coli*, 50 per cent GC; *Proteus morganii*, 52 per cent GC; *Aerobacter aerogenes*, 55 per cent GC; *Serratia marcescens*, 58 per cent GC; *Pseudomonas aeroginosa*, 65 per cent GC; and various vertebrates, all 42 per cent GC. Salmon sperm DNA

TABLE 1

INFLUENCE OF STREPTOMYCINOID ANTIBIOTICS ON STIMULATION OF AMINO ACID INCORPORATION BY DNA

Additions	Cpm Incorporated	
	No DNA	+ 400 μg denatured salmon DNA
None	1,430	1,469
0.1 μg Neomycin	3,890	8,110
1.0 μg Neomycin	6,630	35,200
10 μg Neomycin	5,450	36,300
0.1 μg Streptomycin	...	3,600
1.0 μg Streptomycin	...	4,420
10 μg Streptomycin	...	3,410
1 μg Kanamycin	2,840	6,410
10 μg Kanamycin	3,100	12,910
5 μg Poly U	53,900	
5 μg Poly U + 10 μg Streptomycin	30,700	
5 μg Poly U + 1 μg Kanamycin	22,700	
5 μg Poly U + 1 μg Neomycin	17,500	

The reaction mixtures contained, in 0.5 ml, 25 μmoles NH_4Cl, 6 μmoles $Mg(CH_3COO)_2$, 3 μmoles β-mercaptoethanol, 10 μmoles Tris, pH 7.8, 0.05 μmole GTP, 0.5 μmole ATP, 2.5 μmoles phosphoenolpyruvate Na salt, 10 μg PEP kinase, 1 mg 30*S* protein, 0.01 μmole each of all C^{12} amino acids except phenylalanine, and 0.25 μc C^{14} phenylalanine (360 μc/μmole).

TABLE 2

STIMULATION OF AMINO ACID INCORPORATION BY VARIOUS DNA'S IN THE PRESENCE OF NEOMYCIN

Additions	Cpm incorporated
None	1,420
Neomycin	5,980
50 μg Salmon DNA	1,440
50 μg Salmon DNA + neomycin	26,700
60 μg Mouse DNA	1,670
60 μg Mouse DNA + neomycin	47,900
50 μg Rabbit DNA + neomycin	48,400
50 μg Toad DNA + neomycin	42,600
50 μg f2 RNA	3,440
50 μg f2 RNA + neomycin	11,900

As described in Table 1, 0.5-ml reaction mixtures contained 0.01 μmole each of C^{12} amino acid except phenylalanine, and 0.1 μc C^{14} phenylalanine (360 μc/μmole). Where noted, 5 μg neomycin was also present. All DNA's used were denatured.

was a commercial product of Calbiochem. DNA was denatured by boiling in distilled water for 5 min or by treatment for 1 hr at 60 ° in 0.3 *N* NaOH. The alkali treatment also digests contaminating RNA to mononucleotides.

Deoxyribonuclease I was a crystalline preparation, obtained from Worthington Biochemicals, electrophoretically purified to remove traces of ribonuclease.

Apurinic acid was prepared by adjusting the pH of concentrated solutions of salmon sperm DNA to 1.5 by addition of 1 *N* HCl followed by incubation at 37° for 13 hr. The pH was then adjusted to 7.0 and the nucleic acid repeatedly precipitated from ethanol. The final precipitate was dissolved in water to a concentration of 4 mg/ml after removal of ethanol in a stream of nitrogen. The apurinic acid solution was then boiled for 1 min before use.

Results.—Tables 1 and 2 show that single-stranded DNA from several sources was inactive in stimulating protein synthesis unless a streptomycinoid antibiotic was added to the reaction mixture. It can be seen that streptomycin, neomycin, and kanamycin all greatly accentuated the ability of DNA to stimulate protein synthesis. Neomycin was the most effective of the three antibiotics under these

TABLE 3

EFFECT OF INHIBITORS ON AMINO ACID INCORPORATION STIMULATED BY DENATURED DNA IN THE PRESENCE OF NEOMYCIN

Additions	Cpm incorporated
1. None	770
2. 5 μg Neomycin	2,900
3. 5 μg Neomycin + 5 μg puromycin	357
4. 50 μg Denatured salmon DNA	904
5. 50 μg Denatured salmon DNA + 5 μg neomycin	15,600
6. 50 μg Denatured salmon DNA, 5 μg neomycin, 5 μg puromycin	945
7. 50 μg Denatured salmon DNA, 5 μg neomycin, 50 μg chloramphenicol	1,769
8. 50 μg Denatured salmon DNA, 5 μg neomycin, 2.5 μg actinomycin D	13,100
9. 5 μg Neomycin	2,100
10. 50 μg Denatured *E. coli* DNA	1,240
11. 50 μg Native *E. coli* DNA	1,160
12. 50 μg Denatured *E. coli* DNA + 5 μg neomycin	32,900
13. 50 μg Native *E. coli* DNA + 5 μg neomycin	3,100
14. 50 μg Denatured *E. coli* DNA, 5 μg neomycin, 10 μg deoxyribonuclease	1,860
15. 50 μg Native *E. coli* DNA, 5 μg neomycin, 10 μg deoxyribonuclease	1,900
16. 5 μg Poly U	86,400
17. 5 μg Poly U + 10 μg deoxyribonuclease	90,400

Reaction mixture as in Table 1. Expts. 1–8 contained 0.01 μmole of all C^{12} amino acids and 0.3 μc C^{14} isoleucine (240 μc/μmole). Expts. 9–17 contained 0.01 μmole, all C^{12} amino acids except phenylalanine, and 0.1 μc C^{14} phenylalanine (360 μc/μmole).

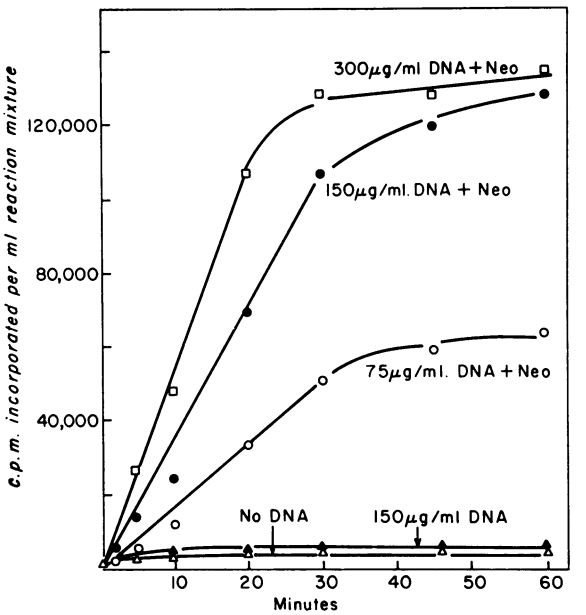

FIG. 1.—Rate of incorporation of C^{14} isoleucine into polypeptide under the influence of denatured salmon DNA. Each 1-ml reaction mixture had the composition given in the legend to Table 1, including 0.02 μmole each C^{12} amino acid and 0.5 μc C^{14} isoleucine (240 μc/μmole). Samples of 0.1 ml were removed at intervals during the incubation at 37°. Neomycin was present in the experiments indicated by the upper three curves at 10 μg/ml and absent in the other two.

conditions, giving optimal effects at about 5 μg/ml.[2] Protein synthesis under the direction of RNA from the *E. coli* bacteriophage f2 was also increased by the presence of neomycin although the effect is not so dramatic.

Table 3 shows that this neomycin-accentuated stimulation of protein synthesis is sensitive to inhibition by puromycin, chloramphenicol, and deoxyribonuclease, indicating that denatured DNA is probably stimulating protein synthesis through mechanisms analogous to those utilized by polyribonucleotide templates. Native *E. coli* DNA has no activity whether or not neomycin is present. On denaturation, the DNA acquires template activity which is completely sensitive to a highly purified deoxyribonuclease. This enzyme preparation has very little ribonuclease activity as evidenced by its failure to inhibit poly U stimulation of phenylalanine incorporation (Table 3). The stimulation by single-stranded DNA is relatively insensitive to actinomycin D, again suggesting that RNA synthesis is not involved in this mechanism. The product of these reactions has the characteristics of normal polypeptide, being sensitive to the proteolytic activity of such enzymes as trypsin and pronase.

Figure 1 illustrates the kinetics of amino acid incorporation stimulated by three different levels of denatured DNA in the presence of neomycin. It can be seen that incorporation is linear for about 30 min and that the degree of stimulation is dependent upon the amount of added DNA. Little stimulation occurred in the presence of DNA or neomycin alone.

In our previous report[1] we showed that only DNA from cell culture sources was

TABLE 4

EFFECT OF STREPTOMYCIN-TREATED DNA ON AMINO ACID INCORPORATION

Additions	Cpm incorporated
1. None	1,420
2. 50 μg Denatured salmon DNA	1,410
3. 50 μg Denatured salmon DNA, streptomycin-treated	12,400
4. Same as 3 with DNA subsequently treated with deoxyribonuclease	3,600
5. Same as 4 with an additional 50 μg denatured salmon DNA added after enzyme treatment	10,000
6. Same as 5 but with 50 μg denatured *E. coli* DNA	13,300
7. 5 μg Poly U	80,200
8. 5 μg Poly U + 50 μg deoxyribonuclease-treated, streptomycin-treated, denatured salmon DNA as in 4	90,000

The denatured salmon DNA was dissolved in H_2O at 1 mg/ml and exposed to streptomycin (1 mg/ml) at room temperature for 30 min and subsequently purified by 10 successive alcohol precipitations. Reaction mixture as in Table 1 containing 0.01 μmole each C^{12} amino acid and 0.1 μc C^{14} phenylalanine (360 μc/μmole). Where noted, DNA's were pretreated with deoxyribonuclease (10 μg/ml) at 37° for 30 min in Tris, 0.01 *M*, $MgCl_2$, 0.01 *M*, and subsequently boiled for 2 min to destroy the enzyme.

capable of marked stimulation of protein synthesis, whereas most DNA was relatively inactive. This difference apparently results from exposure to streptomycin and other aminoglycoside antibiotics during cell growth.[2] Acquisition of template activity may also be effected by exposure of any DNA to a solution of streptomycin. The results of such an experiment are summarized in Table 4. Denatured salmon DNA, ineffective as a template, was mixed with streptomycin and subsequently repurified. The antibiotic is bound to the DNA in this step[6] and the DNA acquires template activity which is sensitive to deoxyribonuclease. Activity may be restored to the deoxyribonuclease digest by the addition of salmon or *E. coli* DNA, presumably by association with streptomycin liberated by the nuclease action. The nuclease digest of streptomycin-treated DNA has no effect on poly U stimulation of C^{14} phenylalanine incorporation.

Figure 2 shows the distribution of acid-insoluble radioactivity after incorporation

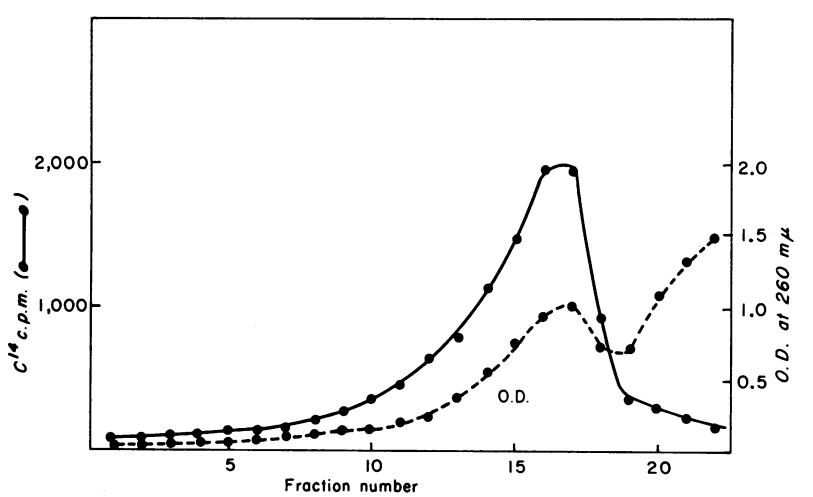

FIG. 2.—Ribosomal location of C^{14} polypeptide product after DNA stimulation of protein synthesis. Reaction mixture as in Table 1 with C^{14} protein hydrolysate. After incubation of the reaction mixture for 1 hr at 37° with 200 μg of salmon DNA and 10 μg of neomycin, 0.15 ml of the reaction mixture was carefully layered on top of a 5–20% sucrose gradient containing 0.1 *M* NaCl; 0.01 *M* Tris·HCl, pH 7.4; 0.015 *M* $MgCl_2$; and centrifuged for 2 hr at 28,000 rpm in the Spinco SW39 swinging bucket rotor. Three-drop fractions collected from the bottom of the tube were analyzed for OD at 260 mμ, then precipitated three times with 5% TCA employing serum albumin as carrier, collected on nitrocellulose filters, and counted.

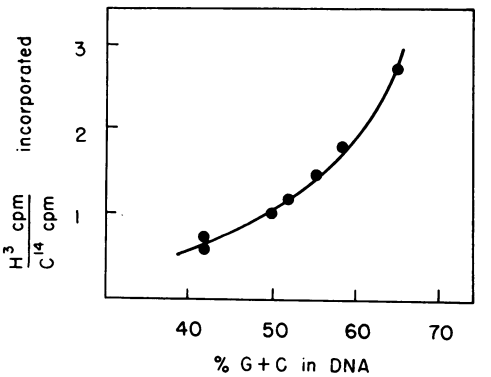

FIG. 3.—Effect of DNA base composition on the ratio of H^3 alanine to C^{14} phenylalanine incorporated into polypeptide. As in Table 1, 0.5-ml reaction mixtures contained 0.01 μmole each of all 20 unlabeled amino acids, 0.1 μc C^{14} phenylalanine (360 μc/μmole), 1.5 μc H^3 alanine (236 μc/μmole), and 25 μg of denatured DNA of different base compositions (see *Materials and Methods*). Results are expressed as the ratio of H^3 cpm to C^{14} cpm incorporated in the presence of a given DNA normalized to the value obtained using *E. coli* DNA (50% GC).

of C^{14} amino acids in a DNA-stimulated cell-free system followed by velocity gradient centrifugation in sucrose. It can be seen that most of the radioactivity is associated with the peak of 70*S* ribosomes. Very little soluble protein was released from the ribosomes and little radioactivity appeared in a polyribosomal region. The absence of polysomes probably reflects the long labeling time, which might allow nuclease scission of the DNA, and the unfavorable stoichiometry between DNA and ribosomes.

Streptomycin can upset the genetic code, causing specific misreadings during polyribonucleotide-stimulated polypeptide synthesis.[7] It is important, therefore, to determine how accurately the genetic code may be read from single-stranded DNA in the presence of streptomycinoid antibiotics. Preliminary tests of this accuracy have been carried out in two ways. According to the work of Sueoka,[8] the incidence of some amino acids in bacterial protein is strongly correlated with the per cent GC in the DNA. The existence of such correlations may also be inferred from the triplet codon assignments of Nirenberg *et al.*[9] If the nucleotide sequence of single-stranded DNA is correctly recognized, the incorporation of these amino acids should be greatly influenced by the composition of the DNA. In Figure 3 the ratio of the amounts of two such amino acids incorporated is shown as a function of the base composition of the DNA. The two amino acids studied were C^{14} phenylalanine, codons UpUpU and UpUpC,[9] and H^3 alanine, codons GpCp(U, C, G, or A).[9] It will be noted that the ratio changes in response to the DNA composition, as is also the case for the protein which such DNA's specify in the living cell.[8]

Another approach involves removal of purines by acid treatment of the DNA and study of the coding ability of the resulting apurinic acid. Table 5 shows that apurinic acid is able to stimulate the incorporation of those amino acids whose known codewords are composed solely of pyrimidines.[9] However, it is nearly inactive as a template for incorporation of amino acids whose known codewords are rich in purines. Presumably, the peptides made are very short, although their high content of phenylalanine would render them acid-insoluble. These results rule out the possibility that the denatured DNA's (or oligodeoxynucleotides) act indirectly in this system through RNA synthesis, for RNA made on pyrimidine oligonucleotides of apurinic acid would be composed exclusively of purines and would therefore give results exactly contrary to those obtained here. These and other experiments[1, 2] provide good evidence that denatured DNA can act directly to stimulate polypeptide synthesis on ribosomes.

TABLE 5

STIMULATION OF AMINO ACID INCORPORATION BY APURINIC ACID

	Cpm Incorporated			
C^{14} Amino acid	Background	+ 200 μg Salmon DNA	+ 400 μg Apurinic acid	Codewords[9]
Alanine	545	13,600	2,010	GCU, GCC, GCA, GCG
Arginine	3,750	57,600	4,800	CGU, CGC, CGA, CGG
Aspartate	377	10,400	776	GAU, GAC
Glutamate	1,240	32,000	3,470	GAA, GAG
Isoleucine	1,120	39,600	2,300	AUU, AUC
Leucine	2,300	40,500	33,600	UUA, UUG, CUU, CUC, CUA, CUG
Phenylalanine	1,420	57,600	23,400	UUU, UUC
Proline	832	29,200	23,500	CCU, CCC, CCA, CCG
Serine	1,740	45,200	28,700	UCU, UCC, UCA, UCG, AGU, AGC
Threonine	850	43,200	776	ACU, ACC, ACA, ACG
Tyrosine	1,430	26,900	4,900	UAU, UAC

Each 0.3-ml reaction mixture, as described in Table 1, contained 5 mμmoles each of all C^{12} amino acids and 1 μc C^{14} alanine (110 μc/μmole), 0.6 μc C^{14} arginine (222 μc/μmole), 1 μc C^{14} aspartate (148 μc/μmole), 1 μc C^{14} glutamate (208 μc/μmole), 1 μc C^{14} isoleucine (222 μc/μmole), 1 μc C^{14} leucine (222 μc/μmole), 1 μc C^{14} phenylalanine (333 μc/μmole), 1 μc C^{14} proline (185 μc/μmole), 1 μc C^{14} serine (120 μc/μmole), 1 μc C^{14} threonine (148 μc/μmole) or 0.5 μc C^{14} tyrosine (375 μc/μmole), and 10 μg neomycin.

Conclusions.—The experiments reported demonstrate that aminoglycoside antibiotics can potentiate the activity of single-stranded DNA as a template for protein synthesis. Whether such a reaction is important as part of the biological activity of such antibiotics remains obscure, although the conversion of DNA into template material inside the living cell could be a lethal event. Certainly these antibiotics have a high affinity for DNA[7] and soluble ribosomes. In particular, 30*S* ribosomes are the sensitive elements[10] whose structure is altered by the mutation to resistance.[11] On the other hand, very little of the cellular DNA is in the single-stranded form necessary for activity, so that intracellular streptomycin would be bound largely to double-stranded DNA.

At the moment it would seem most likely that the *in vitro* effect of the antibiotics depends upon their binding to or influence upon both ribosomes and DNA. Takanami and Okamoto[12] have shown that single-stranded DNA will attach to ribosomes, and it is possible that the antibiotic increases the efficiency of this reaction or promotes the movement of ribosomes along the DNA. Presumably it is attachment of the template to the ribosome and the movement process which is normally forbidden with deoxyribonucleotide polymers. Soluble RNA molecules are capable of recognizing correct codons even when they are part of a polydeoxyribonucleotide. Thus, phenylalanyl-sRNA and lysyl-sRNA are specifically bound to cellulose columns containing polydeoxyribothymidylate and polydeoxyriboadenylate, respectively.[13] The antibiotic may serve the purpose of modification of the site on the ribosome to which the DNA messenger may bind, thus permitting the sRNA to form specific hydrogen bonds with the DNA nucleotides. Recent experiments indicate that ribosomal RNA may also be read in the presence of neomycin.[2]

These findings suggest the feasibility of direct translation of the nucleotide sequences of the gene itself into amino acid sequences. Certainly there is as yet no evidence that such translation is faithful in detail although the available evidence demonstrates at least a grossly sensible reading. Notwithstanding the published results demonstrating that streptomycin can cause a misreading of the code *in vitro*,[7] it might be noted that many investigators have purposely modified conditions in the cell-free synthesizing system to attain maximum misreading.[7, 14] In principle

it may be equally possible to adjust conditions, such as the ionic environment, to allow template activity by single-stranded DNA and preserve specificity. This prediction will be confirmed only when a recognizable peptide or protein can be made directly from the DNA molecule harboring the information for its amino acid sequence. If this hope is realized, this variant of cell-free protein synthesis may offer some advantages by virtue of its high efficiency and the greater ease of fractionating and manipulating DNA as compared with messenger RNA.

Summary.—The activity of some single-stranded DNA in stimulating cell-free protein synthesis previously reported is due to the presence of streptomycin or related antibiotics. The addition of such antibiotics to an *in vitro* system together with single-stranded DNA greatly enhances the incorporation of labeled amino acids. By using DNA of different base compositions and apurinic acid, it is shown that the specificity of amino acid incorporation is consistent with established codeword assignments.

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[1] Holland, J. J., and B. J. McCarthy, these PROCEEDINGS, **52**, 1554 (1964).

[2] Holland, J. J., and B. J. McCarthy, manuscript in preparation (1965).

[3] Berns, K. I., and C. A. Thomas, Jr., *J. Mol. Biol.*, **11**, 476 (1965).

[4] Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961).

[5] Marmur, J., S. Falkow, and M. Mandel, *Ann. Rev. Microbiol.*, **17**, 329 (1963).

[6] Cohen, S. S., and J. Lichtenstein, *J. Biol. Chem.*, **235**, PC 55 (1960).

[7] Davies, J., W. Gilbert, and L. Gorini, these PROCEEDINGS, **51**, 883 (1964).

[8] Sueoka, N., these PROCEEDINGS, **47**, 1141 (1961).

[9] Nirenberg, M., P. Leder, M. Bernfield, R. Brimacombe, J. Trupin, F. Rottman, and C. O'Neal, these PROCEEDINGS, **53**, 1161 (1965).

[10] Spencer, J. H., and E. Chargaff, *Biochim Biophys. Acta*, **51**, 209 (1961); Spotts, C. R., and R. Y. Stanier, *Nature*, **192**, 633 (1961).

[11] Davies, J., these PROCEEDINGS, **51**, 659 (1964); Cox, E. C., J. R. White, and J. G. Flaks, these PROCEEDINGS, **51**, 703 (1964).

[12] Takanami, M., and T. Okamoto, *Biochem. Biophys. Res. Commun.*, **13**, 297 (1963).

[13] Erhan, S., L. G. Northrup, and F. R. Leach, these PROCEEDINGS, **53**, 646 (1965).

[14] So, A. G., J. W. Bodley, and E. W. Davie, *Biochemistry*, **3**, 1977 (1964).