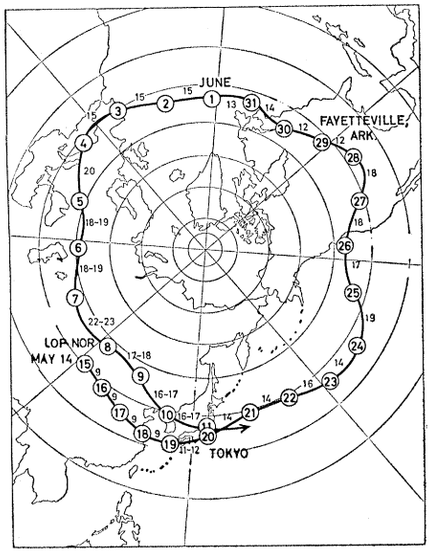

Fig. 2. Air trajectory in the Northern Hemisphere during May and June 1965. This figure indicates the air movement at the height of 5650 meters in the troposphere, which is known to give a representative overall average value of the tropospheric air movement. The numbers in the circles show dates and the numbers between circles show average wind velocities in meters per second.

the movement was about 16 m/sec.

The Sr^{89}/Sr^{90} ratio in rain gradually decreased during the second half of June because of decay of Sr^{89} (half life, 50.4 days), and because of removal of the strontium isotopes from the atmosphere through tropospheric fallout and addition of old debris from the stratosphere. It appears that the Sr^{89}/Sr^{90} ratio will eventually approach a straight line such as $S_o b$, which corresponds to the Sr^{89}/Sr^{90} ratio newly established in the stratosphere. However, a small irregularity was again observed in the middle of July, approximately a month after the 12 June peak occurred. It is probable that this irregularity coincides with the arrival of debris which had by that time circled the world twice and was then beginning its third circuit.

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Amino Acid Changes Provoked by Streptomycin in a Polypeptide Synthesized in vitro

Abstract. *The misincorporation of isoleucine provoked by streptomycin in a polyuridylate-directed incorporating system from* Escherichia coli *has been examined by a double-labeling technique with phenylalanine-^3H and isoleucine-^{14}C. The polypeptides synthesized are associated with 70S ribosomes and contain only phenylalanine in the absence of streptomycin, and both phenylalanine and isoleucine in the presence of the antibiotic. Results of acid hydrolysis and subsequent chymotryptic digestion of the polypeptides indicate that the misincorporated amino acid is inserted by peptide bonding with phenylalanine and is randomly distributed along the chain.*

The ability of streptomycin (Sm) to replace a required growth factor in conditional streptomycin-dependent (CSD) mutants has been interpreted in terms of Sm-activated suppression, whereby a defective protein can be corrected by an amino-acid substitution (*1*). Studies in vitro support this hypothesis, in that Sm provoked the misreading of synthetic polynucleotides in a system containing ribosomes from Sm-sensitive cells with resulting significant misincorporation of amino acids into the material precipitable with trichloroacetic acid (*2, 3*). These studies indicated the ribosome as the site sensitive to streptomycin action and, therefore, implicated the translation process. The sensitivity was later more precisely located as occurring at the step of codon recognition (*4*).

However, the reasonable assumption that this misincorporation is expressed by corresponding changes in the amino acid composition of the resulting polypeptide and the sequence by which the misreading occurs remains to be established directly. We have studied the effect of Sm in the polyuridylate-directed synthesis of polyphenylalanine, which from the incorporation data (*2, 3*) should change from a homopolymer of phenylalanine to a polypeptide consisting of five amino acids. We have used a double-labeling technique with phenylalanine-^3H as the correctly incorporated amino acid and isoleucine-^{14}C as the misincorporated amino acid. This procedure also allows us to follow simultaneously the inhibition of phenylalanine incorporation and the stimulation of isoleucine misincorporation with subsequent analysis of the reciprocal relationship of these two events.

The incorporating system used was derived from *Escherichia coli* (*5*), containing full complement of unlabeled amino acids in addition to the designated labeled ones. From Fig. 1 it can be seen that, as the Sm concentration is increased, the inhibition of phenylalanine incorporation increases and there is a concomitant increase of the misincorporated isoleucine. At a concentration of 2.5 μg of Sm per milliliter the effects were near a maximum, and at 20 μg per milliliter (the usual concentration) the isoleucine replaced 60 percent of the phenylalanine. Moreover, there is an overall stimulation of amino acid incorporation. The degree of stimulation (about 12 percent) deducible from Fig. 1 is a minimum estimate, since only phenylalanine and isoleucine were considered; whereas the contribution from serine, tyrosine, and leucine must also be taken into account.

The study of the polypeptides synthesized in vitro has been carried out on nascent chains bound to ribosomes. Figure 2 shows the sucrose-gradient fractionation of the incubated incorporating system after ribonuclease treatment to destroy messenger RNA. In Fig. 2*a* it is verified that after this treatment phenylalanine (tritium label) remains associated with the 70*S* ribosomal peak (*6*); but more pertinent to this study is the fact that the ^{14}C la-

bel (isoleucine) appeared only when Sm was present in the incubation mixture, and it was associated with the tritium label primarily in the 70*S* ribosomal peak. Some double labeling, irrespective of Sm presence, also appears at the top of the gradient, presumably due to incompletely hydrolyzed sRNA.

Direct evidence for insertion through peptide bonding and evidence on the distribution of isoleucine in the polyphenylalanine chain were sought by chromatographic examination of the products of acid and enzymatic

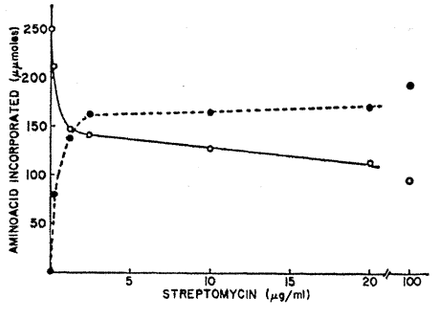

Fig. 1. Effect of streptomycin concentration on phenylalanine and isoleucine incorporation. Ribosome preparation (about 500 μg/ml of incubation mixture) was a dialyzed extract of wild-type *E. coli* B from which natural messenger RNA was removed (*5*). All additions were at the concentrations described (*2*) except that of soluble RNA (from yeast, General Biochemicals), which was 200 μg/ml, and polyuridylic acid (Miles Chemical Co.), which was 80 μg/ml. The buffer was 0.1*M* tris-HCl, *p*H 7.4 containing 0.019*M* magnesium acetate. All amino acids were unlabeled except ^{3}H-phenylalanine (1.5 c/mmole) and ^{14}C-isoleucine (250 mc/mmole). The 0.25-ml samples were incubated at 37°C for 15 minutes, treated with an equal volume of 10 percent trichloroacetic acid, hydrolyzed at 90°C for 20 minutes, and filtered on Millipore filters; the precipitate was washed with 5 percent trichloroacetic acid containing 1 percent Casamino acids, and dried. The ^{3}H and ^{14}C disintegrations were measured simultaneously in a nonaqueous scintillation-counting fluid (Nuclear Chicago Scintillation Counter). The efficiency of counting was 1 percent for ^{3}H and 20 percent for ^{14}C; we took into account that, with the setting used, all tritium counts appeared in one channel, while 20 percent (approximately) of the ^{14}C counts also appeared in this channel. Standards were run with each experiment. The curves for phenylalanine (○) and for isoleucine (●) incorporation were obtained by converting the actual number of counts per minute into $\mu\mu$moles of amino acid (1 $\mu\mu$mole of ^{3}H-phenylalanine being equivalent to 33.2 count/min and 1 $\mu\mu$mole of ^{14}C-isoleucine being equivalent to 11.1 count/min).

hydrolysis of the ribosome-bound nascent polypeptide. Ribosomes carrying radioactive polypeptide were obtained from a 1-ml incorporating mixture, incubated as described (Fig. 1), which was centrifuged at 50,000 rev/min for 4 hours and washed in Nirenberg's buffer A (*7*). The pellet, redissolved in water and precipitated with an equal volume of 10 percent trichloroacetic acid, was incubated at 90°C for 20 minutes. The insoluble material, which consists of ribosomes with attached nascent polypeptides, was then collected by centrifugation and washed with 5 percent trichloroacetic acid.

Attempts to hydrolyze the attached polypeptides by direct enzymatic action were unsuccessful. Evidence for the existence of peptide bonding was therefore sought from the action of chymotrypsin on the shortened peptides obtained by partial acid hydrolysis (in 6*N* HCl at 100°C in sealed ampules for 2½ hours). The chromatography profiles of the radioactive peptides obtained by acid hydrolysis are shown in Figs. 3*a* (Sm absent) and 4*a* (Sm present). From Fig. 3*a* the main products from polyphenylalanine are peptides consisting of at least four amino acid residues. The same procedure on the polypeptide synthesized in the presence of Sm (Fig. 4*a*) yielded more of the free amino acids, presumably because the insertion of other amino acids into the polyphenylalanine results in greater solubility or lability to HCl or both.

The result of subsequent digestion with chymotrypsin (0.125 percent chymotrypsin in 0.01*N* tris-HCl buffer, *p*H 9.0, with 0.001*M* $CaCl_2$ for 3 hours at 37°C) is shown in Figs. 3*b* and 4*b*. A comparison of Fig. 3*a* with Fig. 3*b* reveals a dramatic change in the profile of radioactivity. The extensive decrease in the large-peptide region results in a corresponding high yield in free phenylalanine and in a broad peak of radioactivity in the tripeptide region extending into the dipeptide zone. This is expected, considering that polyphenylalanine consists of a sequence of identical amino acid residues freed specifically by the hydrolysis of their peptide bonds with chymotrypsin. The action of chymotrypsin on the product of incompleted acid hydrolysis of the polypeptide synthesized in the presence of Sm (Fig. 4*a*) is shown in Fig. 4*b*.

The change in radioactivity is of considerable importance in deducing the structure of this polypeptide. There is a reduction of approximately 35 percent for both labels in the large-peptide fraction, accompanied by an increase in both ^{3}H and ^{14}C distributed throughout the di-, tri-, and tetrapeptide regions. No significant increase in the yield of free amino acids is seen.

The interpretation of the results (Fig. 4*b*) must take into account that the polypeptide synthesized in the presence of Sm contains, in addition to phenylalanine, a large proportion of isoleucine, serine, tyrosine, and leucine (Fig. 1, illustrates only isoleucine). Moreover, the mode and specificity of action of chymotrypsin have to be considered. This enzyme is an endopeptidase acting only on peptides having at least four amino acid residues, with its major site of action being on the peptide bond to which an aromatic amino acid contributes the carbonyl group. Comparison of

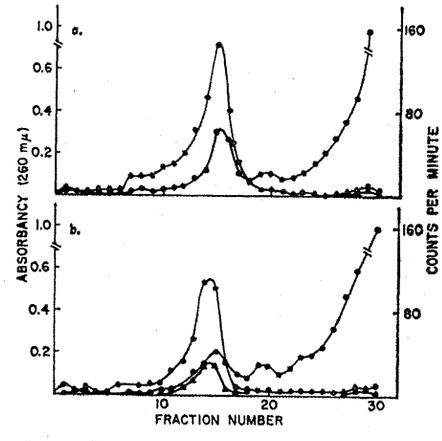

Fig. 2. Effect of streptomycin on the ^{3}H and ^{14}C labeling of sucrose gradient fractions from incorporating systems. The composition of the incorporating system was similar to that described for Fig. 1, except that Sm concentration was zero in (*a*) and 20 μg/ml in (*b*), and ^{14}C-isoleucine was 10 mc/mmole. A portion (1.5 ml) was incubated at 37°C for 15 minutes and treated with ribonuclease (final concentration 7 μg/ml) for 5 minutes at 0°C. Then 1 ml was layered on 25-ml sucrose gradients (5 to 20 percent) (*6*) and centrifuged at 25,000 rev/min for 4 hours. Fractions, two and eight drops alternately, were collected. The absorbancy (●) of the two-drop fraction, after dilution in 0.6 ml water, was read at 260 mμ in a Beckman DU spectrophotometer. Samples (0.1 ml) of the eight-drop fraction were precipitated with trichloroacetic acid, hydrolyzed, and counted for ^{3}H (○) and ^{14}C (▲) as described in Fig. 1. Fraction number denotes paired consecutive two- and eight-drop fractions.

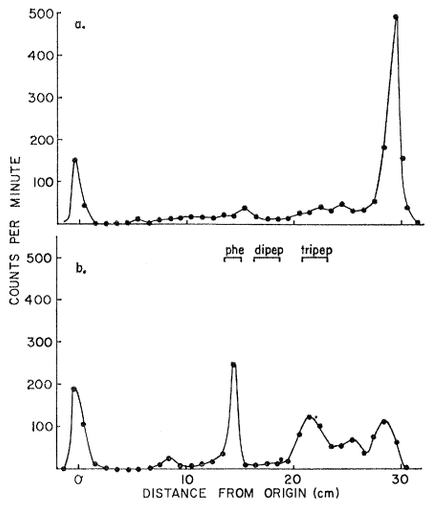

Fig. 3. Paper chromatograms of acid and enzymatic hydrolyzates of polypeptide synthesized in the absence of streptomycin. The solvent system was a mixture of pyridine, isoamylalcohol, and water (35:35:30 by volume) (*12*). Ascending chromatograms were run on Whatman No. 1 paper at room temperature for 15 to 18 hours. The chromatogram was then air dried and sprayed with ninhydrin [0.2 percent (by weight) in *n*-butanol with 4 percent water and 0.6 percent acetic acid (by volume)] to locate reference amino acids and peptides. To locate the radioactive regions, pieces 1 cm long (in the direction of the flow) and 2.5 cm wide were cut from the chromatogram, and the ^{3}H and ^{14}C were determined as in Fig. 1. Reference compounds (used in experiments in Figs. 3 and 4) were: phenylalanine, isoleucine, phenylalanyldipeptide, isoleucylphenylalanine, phenylalanylisoleucine, isoleucyldipeptide (denoted as dipep.), and phenylalanyltripeptide (denoted as tripep.). The radioactivity shows only the ^{3}H (●) of phenylalanine since ^{14}C radioactivity of isoleucine was not significant. (*a*) Product of incompleted acid hydrolysis of ribosome-bound polypeptide. (*b*) Chymotryptic digest of (*a*).

Fig. 3*b* with Fig. 4*b* suggests that no extensive sequences of phenylalanine residues occur in the polypeptide synthesized in the presence of Sm; otherwise a significant amount of free phenylalanine should appear. However, notwithstanding this lack of free amino acid liberation, a small but definite increase of di-, tri-, and tetrapeptides is observed. Therefore it seems reasonable to conclude that the misincorporated amino acid residues are randomly distributed among the phenylalanine residues.

Thus the polypeptide synthesized in the presence of Sm apparently has a certain amount of random replacement of phenylalanine residues by isoleucine. From the misincorporation studies (*2*, *3*) tyrosine, serine, and leucine should be expected to behave similarly (although quantitatively to a lesser degree), and so the polypeptides we studied actually were heteropolymers of five amino acid residues. However, the simple picture of replacement of one reading by another should imply a strict complementarity between correct reading and misreading. Yet the present and previous work (*2*, *3*) indicate an overall stimulatory effect of Sm on the polyuridylate-directed incorporation. This stimulation is moderate with polyuridylate, but it is known (*8*) that it is much more pronounced with polyadenylate and polycytidylate. Since the plateau of amino acid incorporation is practically reached during the routine incubation time, it is fair to assume that the number of formed peptide bonds is smaller in the absence of Sm because of some collateral effect which can be counteracted by the antibiotic. Although possible explanations have been suggested (*8*, *9*) the reasons are far from clear. The information obtained in this work was sought initially with the uridylate system, in which this complication was minimum; polyuridylate has the advantage, compared with the natural and the complex synthetic messengers, of presenting a simple message devoid of signaling complications.

Though polyuridylate is a somewhat atypical messenger, there are similarities between conditions in vivo and those used in our work. The near-maximum effect is observed in our system (Fig. 1) at that concentration of Sm (2.5 μg/ml) which is just sublethal to the cell. This is a good correlation if we accept the fact that lethality is due to extreme misreading. Furthermore, the CSD mutants derived from Sm-sensitive cells exhibited a comparatively greater correction of defective enzymes at drug concentrations of 1 to 3 μg per milliliter than CSD mutants derived from Sm-resistant parents (*10*), which in the same range reveal little misreading (*11*). These findings substantiate the idea that Sm-activated suppression is due to amino acid substitutions. The demonstration in vivo of such an amino acid substitution might well be found by the isolation of temperature-sensitive CSD mutants which, though prototrophic at 25°C, produce a heat stable

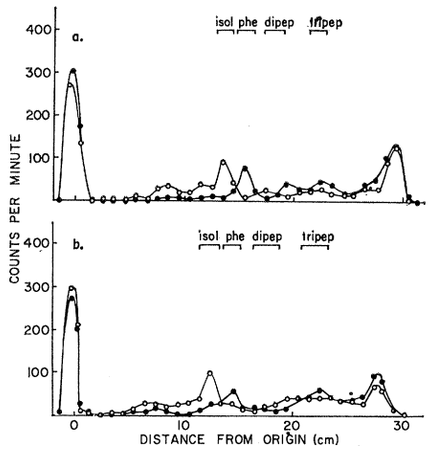

Fig. 4. Paper chromatograms of acid and enzymatic hydrolyzates of polypeptide synthesized in the presence of streptomycin. All conditions as described for Fig. 3, except that Sm (20 μg/ml) was present in the incorporating system. ○, ^{14}C radioactivity; ● for ^{3}H radioactivity. (*a*) Product of incompleted acid hydrolysis of ribosome-bound polypeptide. (*b*) Chymotryptic digest of (*a*).

enzyme at 37°C only in the presence of Sm. Such mutants have now been isolated in our laboratory (*13*).

The randomness of the replacing amino acid throughout the polypeptidic chain synthesized in the presence of Sm agrees with the idea that in any given system the population of ribosomes responds uniformly, and that under the influence of the drug they have the same probability of misreading.

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