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[53] *Note added in proof:* When mitochondrial DNA, which was shown by density gradient centrifugation to be free of nuclear DNA contamination, was heated to 80°C in a lower salt concentration solution of 0.01 × SSC, all the DNA was converted to a symmetrical band of density 1.721.
[54] *Note added in proof:* Recently, mitochondrial DNA has been prepared which shows no demonstrable nuclear contamination by buoyant density centrifugation. The denaturation temperature of this DNA in 0.01 × SSC was 66°C, compared to 61°C for nuclear DNA. Comparison with denaturation temperatures of DNA samples having a wide range of buoyant densities, using the same conditions, gave a G-C content of 47.5% for mitochondrial DNA and 40% for nuclear DNA. This is in good agreement with the values calculated from the buoyant densities. These data indicate that the difference in buoyant density between mitochondrial and nuclear DNA is due to different G-C contents and not to the presence of an unusual or substituted base.

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*AN EFFECT OF STREPTOMYCIN ON THE BIOSYNTHESIS OF THE COAT PROTEIN OF COLIPHAGE f2 BY EXTRACTS OF E. COLI**

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The RNA of f2 bacteriophage directs the synthesis of the viral coat protein in cell-free extracts.[1] The information specifying the amino acid sequence of the protein resides only in the viral nucleic acid.[2]

Gorini and his co-workers have shown that streptomycin (Sm) suppresses mutations in *E. coli*,[3] and changes the amino acid composition of polypeptides whose synthesis is stimulated by synthetic polynucleotides.[4] They proposed that Sm introduces misreadings into the translation of the amino acid code. Valentine and Zinder[5] have reported that host-dependent mutants of f2 bacteriophage, now shown to be defective in the formation of coat protein,[6] were able to form virus particles in nonpermissive bacteria in the presence of Sm. The experiments described here offer evidence that in cell-free extracts Sm modifies the reading of information contained in viral RNA.

Materials and Methods.—Sm sulfate, obtained from Eli Lilly and Co., was not contaminated with amino acids. Bacteriophage f2 was the gift of N. D. Zinder. Tobacco mosaic virus (TMV) was obtained and purified as described previously.[2] The bean form of TMV,[7] purified by M. Rees, was the gift of R. Haselkorn. Viral RNA was prepared and its concentration estimated as before.[1] sRNA purified from *E. coli* B was the gift of R. Wolfenden. Extracts were prepared from *E. coli* K12 substrains K37 and K38[8] by the method of Nirenberg and Matthaei.[9] No differences were found between extracts of the two substrains. After they were harvested during early exponential growth in rich medium,[10] cells were washed and then broken under a pressure of 20,000 psi at −70° in a modified Hughes press (Biochemical Processes, New York, N.Y.). The frozen mass of broken cells was extracted at pH 7.4 with an equal volume of cold, 10 mM Tris HCl buffer containing 11 mM magnesium acetate, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 5 μg/ml DNase (Worthington). After standing for about an hour at 0° the extract was centrifuged at 15,000 × *g* for 10 min. The resulting supernatant was then centrifuged at 30,000 × *g* for 30 min. This supernatant was then preincubated for 30 min with 3 mM ATP, 0.2 mM GTP, 10 mM phosphoenolpyruvate (PEP), 30 μg/ml pyruvate kinase (Boehringer), 20 mM GSH, 11 mM magnesium

acetate, 30 mM KCl, 50 mM Tris HCl buffer, pH 7.8, 1 mM EDTA, 5 μg/ml DNase, and a volume of extract three quarters of the total preincubation volume. No amino acids or sRNA were added. After dialysis at 4° overnight against 10 mM Tris HCl, pH 7.8, 11 mM magnesium acetate, 30 mM KCl, 5 mM 2-mercaptoethanol, and 1 mM EDTA, the extract was stored at −20°.

Before each of the incubation experiments to be described, a portion of the extract was thawed, and again preincubated for an additional 10 min with Tris buffer, GSH, PEP, magnesium acetate, KCl, and pyruvate kinase in a volume slightly less than that finally used; C^{14}-amino acids, viral RNA, and other materials were then added to complete the volume necessary to bring the concentrations of components to those listed for the first preincubation. All incubations were performed at 35°. In some experiments, as indicated, 0.1 *M* NH_4Cl replaced 0.03 *M* KCl. Incorporation of amino acids into protein was estimated as before.[2] Uniformly C^{14}-labeled L-asparagine (30 mC/mmole) was obtained from Nuclear-Chicago; other uniformly labeled L-amino acids were from New England Nuclear. C^{14}-amino acid incorporation was found to be proportional to the amount of S-30 used from 2–9 mg/ml of extract protein; hardly any incorporation occurred when less than 2 mg/ml were used. Protein concentration was measured using bovine serum albumin as a standard.[11]

Preparation of C^{14}-lysine- and C^{14}-arginine-labeled proteins for tryptic hydrolysis, and the methods used for separation and identification of peptides by two-dimensional electrophoresis followed by radioautography have been described before.[1, 2]

Results.—In the absence of supplementary amino acids the addition of Sm to preincubated extracts of *E. coli* enhanced incorporation of a number of C^{14}-amino acids into protein synthesized under the direction of f2 RNA. This effect of Sm is shown in Table 1. Stimulation of protein synthesis was best at a concentration of Sm 0.5–1 μg per ml of reaction mixture. The concentration of Sm which brings about maximal stimulation varied with the amount of extract used in the reaction; it is best expressed as 0.1 μg of Sm per mg of extract protein used. This amount is approximately equal to one molecule of Sm per ribosome. At higher concentrations of Sm, stimulation of amino acid incorporation was less, while further increases were inhibitory (Table 1).

Preincubated extracts in the absence of viral RNA incorporated about 0.02 mμmole/ml of lysine in 1 hr. Sm had no effect on this incorporation. Lysine incorporation increased linearly with the concentration of f2 RNA in the range between 200 and 700 μg RNA per ml; from 700 to 1400 μg/ml, the increase was less. At all concentrations of RNA the addition of Sm was found to stimulate the in-

TABLE 1

EFFECT OF SM ON C^{14}-AMINO ACID INCORPORATION INTO PROTEIN STIMULATED BY f2 RNA

	mμmoles C^{14}-Amino Acid Incorporated/ml				
Sm(μg/ml)	Lys	Arg	Phe	Ala	His
0	1.26	0.24	0.34	0.83	0.59
0.1	1.72	0.28	—	1.03	0.72
0.2	—	—	0.66	—	—
0.5	3.22	0.80	0.89	1.48	1.63
1	4.04	1.36	0.68	1.51	2.01
2	3.17	1.20	0.51	1.21	1.57
5	2.10	0.86	—	0.87	1.05
20	—	—	0.47	—	—
100	0.86	0.60	0.38	0.62	0.77

Incubation mixtures contained 9 mg/ml of extract protein and 700 μg/ml f2 RNA, in a volume of 100 μl, except when C^{14}-phenylalanine was used. With phenylalanine, the mixture contained 5 mg/ml of protein and 560 μg/ml of f2 RNA. After 1 hr of incubation, 50-μl samples were taken for counting. Concentrations and specific activities of the C^{14}-amino acids were as follows: lysine, 22 μM, 220 mC/mmole; arginine, 26 μM, 240 mC/mmole; phenylalanine, 25 μM, 100 mC/mmole; alanine, 40 μM, 122 mC/mmole; histidine, 20 μM, 240 mC/mmole. Incorporation of amino acids in the absence of f2 RNA never amounted to more than 0.05 mμmoles; this incorporation has been subtracted.

corporation of lysine by the same factor (Fig. 1). This factor was greatest when the incubation was performed in KCl; in NH_4Cl no stimulation was seen at 60 min. Stimulation was also more prominent during the early phase of the reaction. Thus in KCl, Sm increased sevenfold the amount of lysine incorporated during 10 min; at 1 hr the stimulation was only threefold.

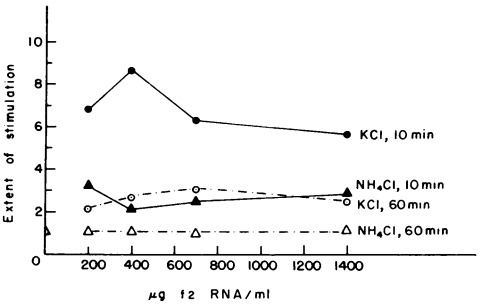

FIG. 1.—Incubation mixtures with either 0.03 *M* KCl or 0.1 *M* NH_4Cl contained 9 mg/ml of extract protein in a volume of 100 μl. f2 RNA was added in the concentrations indicated. The concentration of lysine was 22 μM and its specific activity was 110 mC/mmole. The concentration of Sm was 1 μg/ml. After 10 and 60 min of incubation, 20-μl samples were taken for counting. The ratio of lysine incorporated in the presence of Sm to that amount in its absence (*extent of stimulation*) is shown as a function of f2 RNA concentration. A ratio of 1 as obtained with NH_4Cl at 1 hr indicates no stimulation by Sm. At 1 hr in the absence of f2 RNA lysine incorporation was 0.02 mμmole in KCl and 0.04 mμmole in NH_4Cl. Subtraction of these values would not significantly change the factor of stimulation.

The time course of lysine incorporation in KCl is presented in Figure 2*a*. Again the effect of Sm was greatest at the start of the incubation and diminished at later times. This feature of the stimulation was most obvious when the time course was studied in NH_4Cl (Fig. 2*b*). When 0.1 *M* NH_4Cl was used in place of 0.03 *M* KCl, Sm increased the initial rate of incorporation threefold. Nevertheless, the final amount of lysine incorporated with Sm was only 1.2 times greater than that incorporated in its absence.

The observed kinetics indicate that the concentration of an unknown component in the reaction mixture limits the rate of lysine incorporation, but not the final amount incorporated, and that Sm stimulates by relieving this limitation. It was found that supplementation of the extracts with C^{12}-amino acids enhanced C^{14}-amino acid incorporation stimulated by f2 RNA (Fig. 3). Incorporation of C^{14}-lysine depended upon the addition of C^{12}-amino acids in KCl. Little or no dependence upon supplemental amino acids was seen when the incubation was performed in NH_4Cl. Addition of sRNA did not affect incorporation in extracts unsupplemented with amino acids in either the presence or absence of Sm.

Various groups of C^{12}-L-amino acids were tested for their effect on C^{14}-lysine incorporation under conditions where the addition either of Sm or of all the amino acids would stimulate protein synthesis (Table 2). Asparagine was the amino acid which most stimulated the rate of C^{14}-lysine incorporation. Addition of asparagine increased the rate 5 times, as did the addition of Sm. Apparently the concentrations of other amino acids also limited the rate of protein synthesis, since all the amino acids, when added together, stimulated more than did asparagine alone. Of the five groups of amino acids tested, only group I, which contained asparagine, stimulated when added by itself. Similar results were obtained when C^{14}-phenylalanine was used as the tracer amino acid instead of lysine. The rate of protein synthesis was found to depend upon the concentration of asparagine up to 0.1 mM.

Stimulation of protein synthesis by Sm depended on the template RNA used. When the RNA either of TMV or of bean virus was added to extracts of *E. coli*

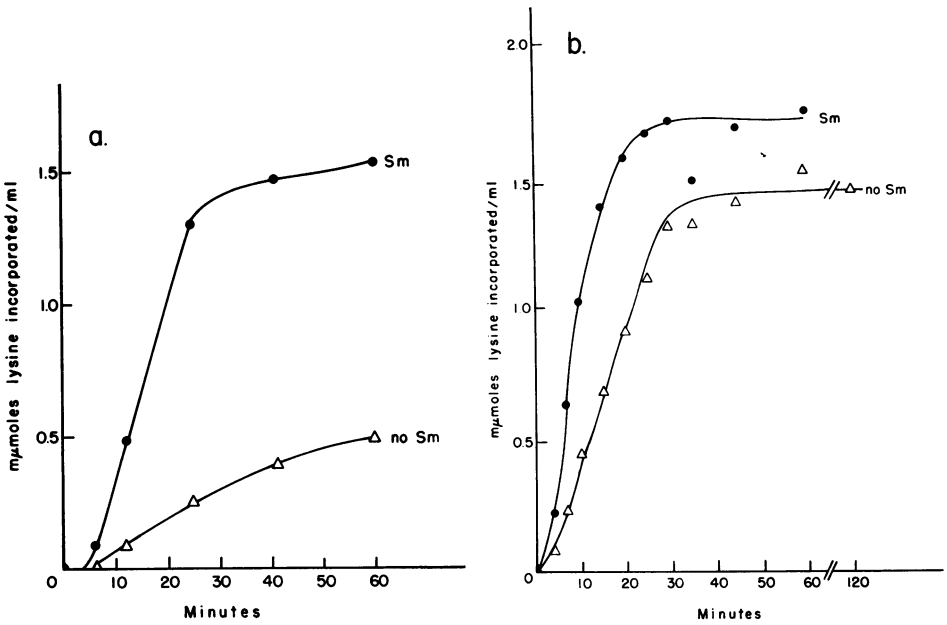

FIG. 2.—(*a*) Incubation mixtures contained 0.03 *M* KCl, 5 mg/ml extract protein, and 560 μg/ml f2 RNA in a volume of 100 μl. The concentration of lysine was 55 μM and its specific activity was 110 mC/mmole. (*b*) Incubation mixtures contained 0.1 *M* NH_4Cl, 9 mg/ml extract protein, and 400 μg/ml f2 RNA in a volume of 250 μl. The concentration of lysine was 42 μM and its specific activity was 110 mC/mmole. Samples of 10 or 20 μl were taken for counting at the times indicated. The concentration of Sm was 1 μg/ml. Incorporation of lysine in the absence of f2 RNA was negligible.

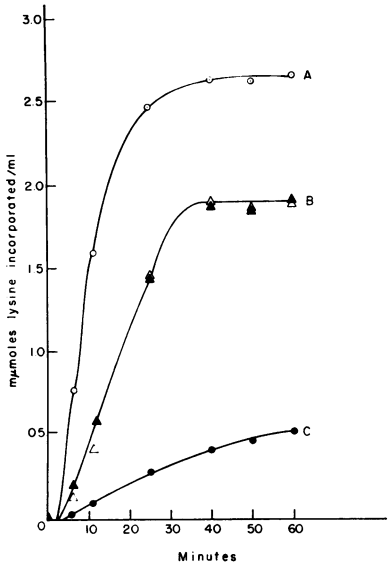

FIG. 3.—Incubation mixtures either with 0.03 *M* KCl or 0.1 *M* NH_4Cl contained 5 mg/ml of extract protein and 560 μg/ml f2 RNA in a volume of 100 μl. The concentration of lysine was 48 μM, and its specific activity was 110 mC/mmole. Time courses of lysine incorporation are (*A*), in 0.03 *M* KCl when all of the C^{12}-amino acids listed in the legend of Table 2 were added, each at a concentration of 0.1 mM; (*B*) (open triangles), in 0.1 *M* NH_4Cl without addition of amino acids; (*B*) (closed triangles), in 0.1 *M* NH_4Cl when all the amino acids were added, each at a concentration of 0.1 mM; and (*C*), in 0.03 *M* KCl without the addition of amino acids. Incorporation of C^{14}-lysine in the absence of f2 RNA either in the presence or absence of C^{12}-amino acids was negligible.

unsupplemented with amino acids, asparagine did not limit C^{14}-lysine incorporation, and Sm did not stimulate protein synthesis. On the contrary, as shown in Table 3, Sm inhibited the rate of lysine incorporation.

Biosynthesis of protein is thought to be a sequential process in which amino

acids are incorporated one after the other in an obligatory order. The lack of any amino acid whose incorporation should occur early in the synthesis of the protein would block incorporation of other amino acids. Furthermore, synthesis of the polypeptide chain should proceed up to the position where incorporation of the lacking amino acid is expected, and there stop. After a time of synthesis, addition of the missing amino acid should result in rapid completion of the protein. In extracts unsupplemented with amino acids, protein synthesis proceeded at a slow rate due to deficiency of asparagine. Protein synthesis was not completely blocked, perhaps because asparagine may be generated slowly in the extracts; enzymatic formation of asparagine and possibly of other amino acids is suggested by the stimulatory effect of NH_4Cl in extracts unsupplemented with amino acids (Fig. 3). Nevertheless, amino acid incorporation was considerably slowed, and the foregoing considerations predict a restoration of the rate of protein synthesis upon addition of asparagine. It was found that addition of asparagine or Sm during incubation of unsupplemented extracts in the presence of f2 RNA resulted in stimulation of C^{14}-lysine incorporation without lag, at rates greater than or equal to that seen when asparagine or Sm had been added at the start of the incubation. An experiment using Sm to stimulate protein synthesis is shown in Figure 4; similar results were obtained when asparagine was added instead of Sm. These results indicate not only that the incorporation of

TABLE 2

EFFECT OF C^{12}-AMINO ACIDS AND SM ON THE RATE OF C^{14}-LYSINE INCORPORATION

Addition	mμmoles Lysine incorporated/ 7 min/ml
None	0.07
I	0.46
II	0.11
III	0.09
IV	0.09
V	0.10
All amino acids	0.95
Asn	0.36
Gln	0.10
Arg	0.09
His	0.07
Sm	0.39

Incubation mixtures contained 9 mg/ml of extract protein and 700 μg/ml of f2 RNA in a volume of 50 μl. Amino acids and groups of amino acids were added so that the concentration of each amino acid was 0.1 mM. The groups contained the following L-amino acids: I, asparagine, arginine, histidine, and glutamine; II, aspartic acid, glutamic acid, methionine, and glycine; III, serine, threonine, and cysteine; IV, valine, leucine, isoleucine, and alanine; V, phenylalanine, proline, tyrosine, and tryptophan. The concentration of lysine was 42 μM, and its specific activity was 110 mC/mmoles. The concentration of Sm was 1 μg/ml. Twenty-μl samples were taken for counting. Incorporation of lysine in the absence of f2 RNA was less than 0.02 mμmoles, and was not subtracted.

TABLE 3

EFFECT OF SM ON THE INCORPORATION OF C^{14}-LYSINE DIRECTED BY TMV AND BEAN VIRUS RNA

Addition	mμmoles Lysine Incorporated/ 12 Min/ml	
	TMV	Bean virus
None	0.08	1.07
Sm	0.01	0.93
Asn	0.07	1.45
All amino acids	0.06	2.48

Incubation mixtures contained 5 mg/ml of extract protein and 240 μg/ml viral RNA in a volume of 100 μl. The concentration of lysine was 48 μM and its specific activity was 220 mC/mmole. C^{12}-asparagine and all the amino acids listed in the legend of Table 2, when added, were each at a concentration of 0.1 mM. The concentration of Sm was 1 μg/ml. During the linear phase of the reaction, 20-μl samples were taken for counting. Incorporation of lysine in the absence of viral RNA was less than 0.01 mμmole, and was not subtracted.

TABLE 4

EFFECT OF SM ON C^{14}-LYSINE INCORPORATION IN THE PRESENCE OF ALL C^{12}-AMINO ACIDS

Addition	mμmoles Lysine incorporated/ 25 min/ml
None	0.26
All amino acids	2.78
Sm	1.30
Sm and all amino acids	1.04

Incubation mixtures and experimental conditions were described in the legend of Fig. 2a. All the amino acids listed in the legend of Table 2 were added, each at a concentration of 0.1 mM.

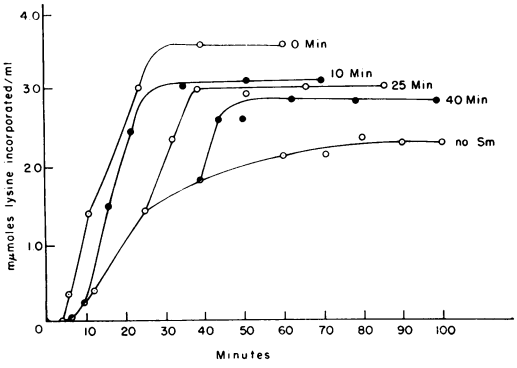

FIG. 4.—A mixture containing 5 mg of extract protein and 560 μg of f2 RNA in 1 ml was incubated for 100 min. During the course of the incubation period, at 0, 10, 25, and 40 min after the start of the reaction, 1 μg/ml of Sm was added to 100-μl portions of the original mixture. The time course of lysine incorporation was determined by removing 10- or 20-μl samples from these portions at 6, 12, 25, 40, and 60 min after the addition of Sm. Incorporation occurring in the original mixture in the absence of Sm was also measured during the 100 min of the incubation as indicated. Incorporation of lysine in the absence of f2 RNA was negligible. The concentration of lysine was 48 μM and its specific activity was 110 mC/mmole.

lysine was limited by the availability of asparagine but also that it was contingent upon the prior incorporation of asparagine.

The time course of C^{14}-asparagine incorporation into protein in the presence of f2 RNA is shown in Figure 5. Asparagine was rapidly incorporated without lag. Addition of C^{12}-lysine did not alter the rate or the final amount of C^{14}-asparagine incorporated, nor did a fourfold excess of C^{12}-aspartic acid. C^{14}-lysine incorporation was studied in parallel. After a lag of 6–8 min, C^{14}-lysine was slowly incorporated. C^{14}-lysine incorporation, as before, was stimulated by addition either of C^{12}-asparagine or of Sm. Furthermore, as shown in Figure 5, Sm depressed by half the incorporation of C^{14}-asparagine. The inhibitory effect of Sm probably reflects a general retardation of protein synthesis, and was not due specifically to misreading of the codon for asparagine. This additional effect was suggested by the observation that high concentrations of Sm inhibited amino acid incorporation (Table 1), and further supported by the fact that addition of Sm and of all the amino acids did not result in a greater rate of C^{14}-lysine incorporation than did the addition of Sm alone (Table 4). Similar results were also found when C^{14}-phenylalanine incorporation was used as a measure of protein synthesis.

Discussion and Conclusions.—The sequence of amino acids in protein is determined by information encoded in template RNA. Both RNA and protein are linear molecules; the linear character of the information in RNA and in the protein product implies that the translation of information is a sequential process. Protein synthesis is believed to occur on ribosomes to which template RNA and peptidyl-sRNA are bound. Incorporation of an amino acid is achieved by transferring the peptidyl group from its sRNA to the amino group of the adding aminoacyl-sRNA.[12] Dintzis demonstrated that in rabbit reticulocytes hemoglobin was synthesized from its amino terminus to its carboxy terminal end.[13] Thus as one aminoacyl-sRNA joins the growing peptide chain, a requirement develops for another. The lack of an amino acid which occurs in the polypeptide chain near the amino terminus of the protein would block incorporation of other amino acids.

Deficiency of asparagine in extracts was due to its destruction during incubations. Asparagine was completely converted to aspartic acid within 2 min under conditions used for the incorporation of C^{14}-asparagine into protein (Fig. 5). Sm had no effect on this conversion. Asparaginase has been found in extracts of *E. coli.*[14]

Sm stimulated amino acid incorporation only under conditions where the concentration of asparagine limited protein synthesis. The effect of Sm was diminished or absent in NH_4Cl; this suggests that ammonium ion was used in extracts for the generation of asparagine. Furthermore, in the absence of bacteriophage RNA or with a viral RNA in which incorporation of lysine did not depend upon the concentration of asparagine, Sm did not stimulate (Table 3). Proportional increase in the rate of amino acid incorporation with increasing concentrations of f2 RNA may be presumed to arise from an increase in the number of sites of protein synthesis. The factor of stimulation by Sm was not affected by the concentration of f2 RNA (Fig. 1), and this suggests that Sm stimulates protein synthesis by bringing about more efficient use of existing sites. A reasonable explanation for the effect of Sm is suggested by its action as a suppressor of mutant bacterial[3] and viral[5] functions. When, during protein synthesis, it becomes obligatory that an asparagine codon be translated, Sm can stimulate amino acid incorporation by causing a misreading of that codon. Misreading of the asparagine codon would permit the use of some other amino acid whose concentration was not limiting.

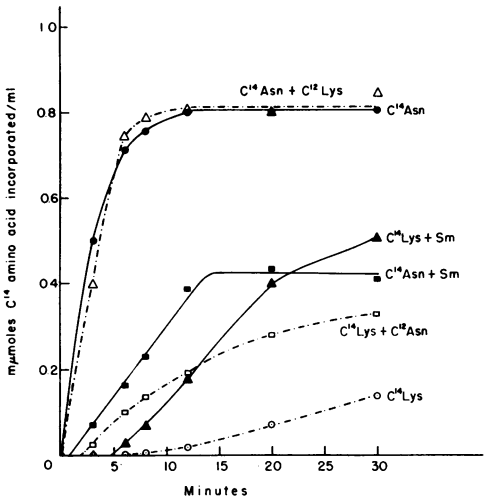

FIG. 5.—Incubation mixtures contained 5 mg/ml of extract protein and 560 μg/ml of f2 RNA in 250 μl. Additions were made as indicated in the figure. C^{14}-asparagine was added at a concentration of 50 μM, and lysine at 48 μM and with a specific activity of 110 mC/mmole. C^{12}-lysine or C^{12}-asparagine were added at concentrations of 50 μM. The concentration of Sm was 0.75 μg/ml. C^{14}-amino acid incorporation was determined by removing 50-μl samples after 3 min of incubation and 20-μl samples at the times indicated thereafter. Incorporation of C^{14}-amino acids in the absence of f2 RNA was negligible.

Substitution of another amino acid for asparagine would probably occur early in the synthesis of the protein. Peptide maps of material synthesized in the presence of Sm show that it stimulates the incorporation of C^{14}-lysine and C^{14}-arginine into coat protein peptides. No differences were seen in the pattern of soluble, tryptic peptides from products made in the presence or absence of Sm. This result is expected since there is evidence that the amino terminal tryptic peptide is insoluble and contains over half the possible asparagine residues of the coat protein.[15] Differences have been seen in peptides from chymotryptic hydrolysates labeled with C^{14}-phenylalanine; these are now under investigation.

The rate of amino acid incorporation was found to depend upon the concentration of asparagine or the presence of Sm. That the incorporation of one amino acid is dependent upon the prior incorporation of another is a consequence of the sequential nature of protein synthesis. These experiments illustrate the feasibility of the proposal that the rate of protein synthesis might be regulated by the availability of particular aminoacyl-sRNA's.[16]

The author wishes to thank Norton D. Zinder for his contribution to this work, which, apart from

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*ASYMMETRIC RNA SYNTHESIS IN VITRO: HETEROLOGOUS DNA-ENZYME SYSTEMS; E. COLI RNA POLYMERASE**

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The synthesis of RNA in living cells is so restricted and regulated as to produce patterns of gene expression characteristic of the organism, its time, and its environment. An aspect of this regulation which has received some attention during the past three years is that of strand selection.[1-14]

Recently, some of us described the ability of *B. megatherium* extracts to use selectively one strand of the DNA of its bacteriophage α as a template for RNA synthesis, excluding the other strand.[14] The RNA produced in this *in vitro* system is asymmetric. Its interactions with αDNA and the purified purine-rich ("light") strand of αDNA are the same as those of pulse-labeled RNA from phage α-infected *B. megatherium*. We had shown that the complete integrity of phage DNA is not essential for such asymmetric synthesis, but that its ordered helical conformation is. In this communication we present further properties of this and related