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CHARACTERISTICS AND STABILIZATION OF DNAASE-SENSITIVE PROTEIN SYNTHESIS IN E. COLI EXTRACTS

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It has been assumed for many years that in protein synthesis the base sequence of DNA specifies the base sequence of RNA and that RNA in turn controls the amino acid sequence of protein. In accord with this notion, several groups recently have observed an inhibition of amino acid incorporation into protein by DNAase in cell-free extracts.[1–3] One object of the present investigation was to study this phenomenon further.

A major difficulty in the study of cell-free protein synthesis in *E. coli* systems has been the necessity for preparing fresh enzyme extracts for each experiment. Techniques have not been available for stabilization and storage of enzyme extracts comparable to the techniques available for mammalian systems.[4] In the present communication, an amino acid–incorporating system stable to storage for several months will be described. The characteristics of amino acid incorporation into protein by the stored extracts were investigated also. A part of these data has been presented in a preliminary report.[2]

Methods and Materials.—*E. coli* W3100 cells, harvested in early log phase, were washed by centrifugation and disrupted by grinding with twice their wet weight of alumina A301 (Aluminum Corporation of America) for 5 min at 5°. All subsequent steps were performed at this temperature. The enzymes were extracted with buffer containing 0.01 *M* Tris(hydroxymethyl)aminomethane, pH 7.8; 0.01 *M* magnesium acetate; 0.06 *M* KCl; and 0.006 *M* mercaptoethanol (standard buffer) equivalent to two or three times the wet weight of cells. The extract was centrifuged three times at 30,000 × *g* for 20, 20, and 60 minutes, respectively. The pellets were discarded after each centrifugation. The final supernatant fluid (S-30) was centrifuged at 105,000 × *g* for 2 hr in the Spinco Model L ultracentrifuge to sediment the ribosomes. The supernatant solution (S-100) was aspirated, and the ribosomes were suspended in standard buffer by gentle homogenization in a Potter-Elvehjem homogenizer and were washed by centrifuging again at 105,000 × *g* for 2 hr. The supernatant fluid was decanted and discarded, and the ribosomes (W-Rib) were suspended in the original volume of standard buffer. Fractions S-30, S-100, and W-Rib were dialyzed against 60 volumes of standard buffer overnight at 5° and were stored in small aliquots at − 15° until needed.

DNAase I, RNAase, and trypsin were crystalline preparations obtained from the Worthington

Biochemical Co. The sodium salt of polyadenylic acid (Batch BC-5815-2, Miles Chemical Co.) had a molecular weight of approximately 30,000. U-C^{14}-L-valine (6.5 mC/mM) was obtained from Nuclear-Chicago Corp. Polyglucose carboxylic acid (molecular weight approximately 30,000) was a gift from Dr. Peter Mora.[5] Puromycin was a gift obtained from Dr. Arthur Weissbach. Chloramphenicol was obtained from Parke Davis & Co., and highly polymerized salmon sperm DNA from California Foundation for Biochemical Research.

Proteolytic enzymes as contaminants of the DNAase were assayed by the method of Anson[6] using denatured hemoglobin (Nutritional Biochemical Co.) as the substrate. RNAase contamination of DNAase was assayed by incubation of DNAase with purified RNA, precipitation with cold trichloroacetic acid, and determination of the absorbancy at 260 mμ of the supernatant solution obtained after centrifugation.

The DNAase digest of DNA was prepared by incubating 5 mg/ml salmon sperm DNA with 10 μg/ml DNAase for 6 hr at 35° in 20 μmoles/ml phosphate buffer, pH 7.0, and 20 μmoles/ml magnesium acetate. DNAase was destroyed by three deproteinizations according to the method of Sevag,[7] and traces of solvents were removed by bubbling N_2 through the solution.

Protein was determined by a micro modification of the method of Lowry.[8] The synthetic amino acid solution used contained 1 μmole/ml of each of the following L-amino acids: glycine, alanine, serine, aspartic acid, asparagine, glutamic acid, glutamine, isoleucine, leucine, cysteine, cystine, histidine, tyrosine, tryptophan, proline, threonine, methionine, phenylalanine, arginine, and lysine. The complete reaction mixture is shown in the legend for Table 1.

TABLE 1

CHARACTERISTICS OF C^{14}-L-VALINE INCORPORATION INTO PROTEIN BY CELL-FREE, *E. coli* ENZYME PREPARATIONS STORED FOR SEVERAL WEEKS AT −15°

Experiment no.	Additions	Counts/min/mg protein
1	Complete	679
	− 105,000 × *g* supernatant solution	74
	− Ribosomes	15
	− ATP, PEP, PEP kinase	38
	+ 10 μg RNAase	9
	− Amino acid mixture	365
	+ 0.02 μmole Puromycin	38
	+ 0.30 μmole Chloramphenicol	82
	− 0.03 μmole GTP, CTP, UTP	583
	− 0.03 μmole CTP, UTP	652
	Deproteinized at zero time	4
2	Complete	2078
	+ 10 μg DNAase	1223
	Deproteinized at zero time	5

The reaction mixtures contained the following in μmole/ml: 100 Tris (hydroxymethyl)aminomethane, pH 7.8; 10 magnesium acetate; 50 KCl; 6.0 mercaptoethanol; 1.0 ATP; 5.0 phosphoenolpyruvate, K salt; 20μg phosphoenolpyruvate kinase, crystalline; 0.05 each of 20 L-amino acids minus valine; 0.03 each of GTP, CTP, and UTP; 0.015 C^{14}-L-valine (~70,000 counts); 1.0 and 0.7 mg S-100 and W-Rib protein, respectively were present per reaction mixture in Experiment 1. 2.1 mg S-30 protein were present in Experiment 2. Total volume was 1.0 ml. Samples were incubated at 35° for 60 min, were deproteinized with 10 per cent trichloroacetic acid, and the precipitates were washed and counted by the method of Siekevitz.[13]

Planchets were counted in a Nuclear-Chicago gas flow counter with a Micromil window and a counting efficiency of approximately 30 per cent. All assays were performed in duplicate.

Results.—Stabilization of cell-free extracts: The effects of dialysis and freezing upon the ability of the S-30 fraction to incorporate C^{14}-L-valine into protein are presented in Figure 1. No enzymatic activity was lost after overnight dialysis. If mercaptoethanol was omitted from the dialyzing buffer, rapid loss of activity was observed. Reduced glutathione was not quite as effective as mercaptoethanol in preventing loss of activity.

Dialyzed S-30 fractions were divided into aliquots and were stored at −15°. The results of Figure 1 demonstrate that the enzyme, after storage for twenty-four hr at −15°, was as active as fresh S-30. Frozen preparations lost less than 10 per cent activity per month.

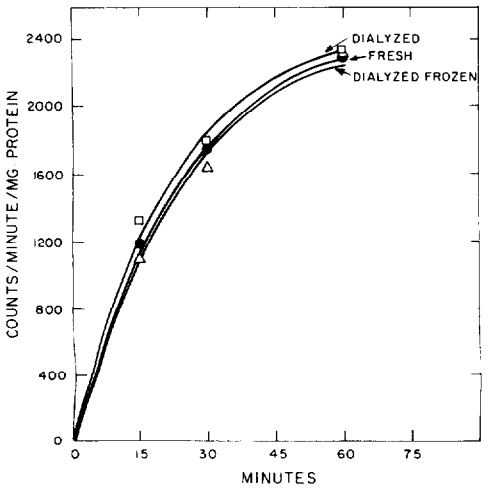

FIG. 1.—The effects of dialysis and freezing upon C^{14}-L-valine incorporation into protein in 30,000 × *g* supernatant layer fractions (S-30). ● Fresh S-30; □ S-30 after 12 hr of dialysis; △ S-30 after dialysis and storage at −15° for 24 hr. The composition of the reaction mixtures is presented in Table 1. 2.1 mg S-30 protein were added to each reaction mixture.

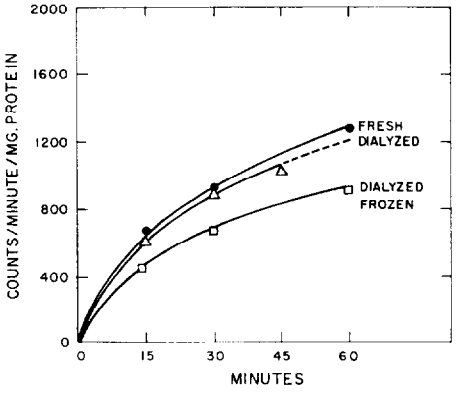

FIG. 2.—The effects of dialysis and freezing upon C^{14}-L-valine incorporation into protein using washed ribosomes (W-Rib) and 105,000 × *g* supernatant solutions (S-100). ● Fresh W-Rib and S-100; △ W-Rib and S-100 dialyzed separately for 12 hr; □ W-Rib and S-100 after dialysis and storage separately at −15° for 24 hr. The composition of the reaction mixtures is presented in Table 1. 0.9 and 1.0 mg W-Rib and S-100 protein respectively were present in each reaction mixture.

When washed ribosomes (W-Rib) and 105,000 × *g* supernatant fluid (S-100) were stored separately or recombined at −15°, some activity was lost compared to the S-30 fraction (Fig. 2). No loss in enzymatic activity of fractions S-100 or W-Rib was observed after overnight dialysis. Again, addition of mercaptoethanol prevented rapid inactivation. Storage of the fractions separately at −15° resulted in a loss of approximately 25 per cent of the activity. The enzyme fractions lost less than 5 per cent of their activity per week, and fractions stored for several months were routinely used in these studies. Figures 1 and 2 also demonstrate that of the total incorporation obtained after incubation for one hour, approximately 50 per cent occurred within the first 15 min.

The characteristics of C^{14}-L-valine incorporation into protein by S-100 and W-Rib fractions stored at −15° for several weeks are presented in Table 1. The rate of incorporation in the absence of either S-100 or W-Rib fractions was negligible. Experiments of this type strongly suggested that W-Rib fractions were not contaminated with intact cells, and this check was routinely performed with each enzyme preparation.

Effects of additions and deletions: Incorporation was dependent upon addition of ATP and an ATP-generating system, and incorporation was completely inhibited by the addition of RNAase. Omitting a mixture of 20 L-amino acids from the reaction mixture resulted in a 46 per cent decrease in incorporation of C^{14}-valine into protein, suggesting *de novo* synthesis of protein. The dependence of C^{14}-L-valine incorporation upon addition of the amino acid mixture was observed only when well-dialyzed preparations were used. Addition of 0.02 μmoles puromycin or 0.30 μmoles chloramphenicol/ml to the reaction mixture depressed amino acid incorporation. Puromycin was a better inhibitor of C^{14}-valine incorporation

than chloramphenicol. Omission of guanosine-5′-triphosphate (GTP), cytidine-5′-triphosphate (CTP), and uridine-5′-triphosphate (UTP) resulted in a slight inhibition of C^{14}-valine incorporation. However, addition of GTP alone largely replaced the mixture of three triphosphates. Experiment 2, also in Table 1, demonstrates that addition of 10 μg of crystalline DNAase markedly inhibited C^{14}-valine incorporation. Further experiments dealing with this effect will be discussed later.

That the incorporation of C^{14}-valine into protein depends upon the presence of the S-100 fraction is further documented in Figure 3. Little C^{14}-valine was incorporated into protein when 0.7 mg W-Rib protein alone was used. The incorporation was proportional to the amount of S-100 fraction added up to 2.5 mg S-100 protein.

In Figure 4 are presented data demonstrating the dependence of C^{14}-valine

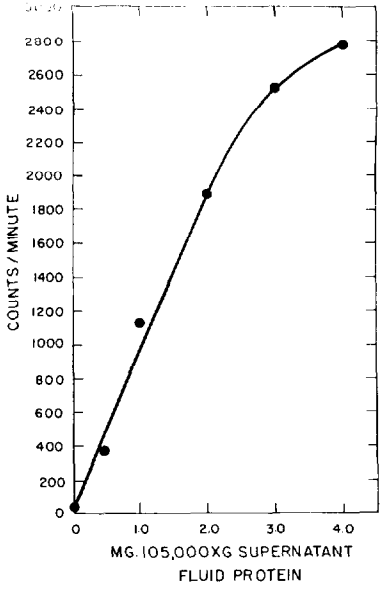

FIG. 3.—The dependence of C^{14}-L-valine incorporation into protein upon the amount of 105,000 × g supernatant solution (S-100). The composition of the reaction mixtures is presented in Table 1. 0.7 mg ribosome protein (W-Rib) were added to each sample. Reaction mixtures were incubated for 60 min at 35°.

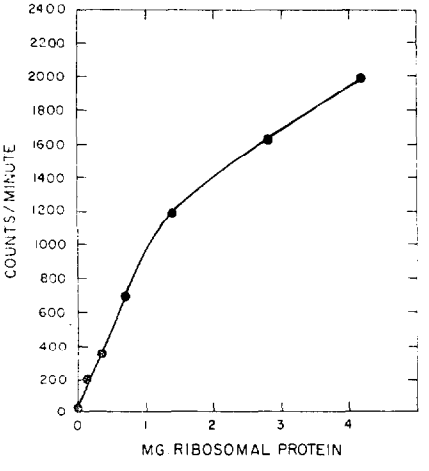

FIG. 4.—The dependence of incorporation of C^{14}-L-valine into protein upon *E. coli* ribosome concentration. The composition of the reaction mixtures is presented in Table 1. 1.0 mg 105,000 × g supernatant solution protein (S-100) was added to each sample. Reaction mixtures were incubated for 60 min at 35°.

incorporation upon W-Rib fractions. No incorporation by 1.0 mg S-100 protein alone was observed. C^{14}-valine incorporation was proportional to the amount of W-Rib added within the range of 0–1.0 mg ribosomal protein.

The effect of pH upon C^{14}-L-valine incorporation into protein is presented in Figure 5. A sharp pH optimum was observed with maximal incorporation at pH 7.8.

DNAase effect: The effect of DNAase upon C^{14}-valine incorporation over a 90-min incubation period is presented in Figure 6. In the absence of DNAase,

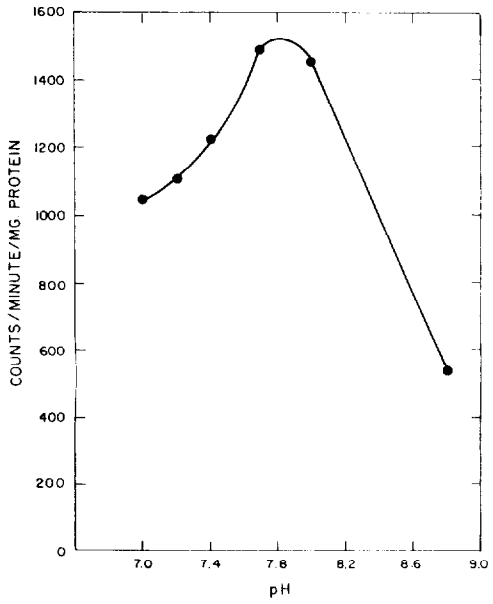

FIG. 5.—The effect of pH upon C^{14}-L-valine incorporation into protein. The composition of the reaction mixtures is presented in Table 1. 100 μmoles Tris(hydroxymethyl)-aminomethane were present in each reaction mixture. Samples were incubated for 10 min at 35°.

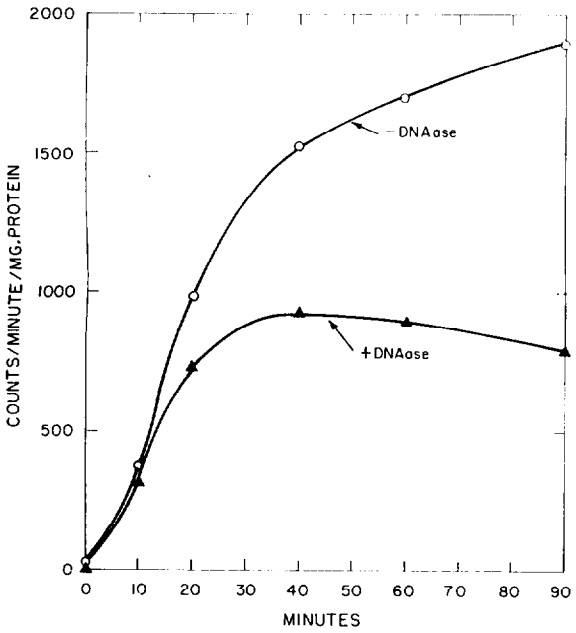

FIG. 6.—The effect of DNAase upon C^{14}-L-valine incorporation into protein. ○ Minus DNAase, △ + 10 μg DNAase. 2.1 mg 30,000 × g supernatant solution protein (S-30) were present in each reaction mixture. The composition of the reaction mixtures is presented in Table 1.

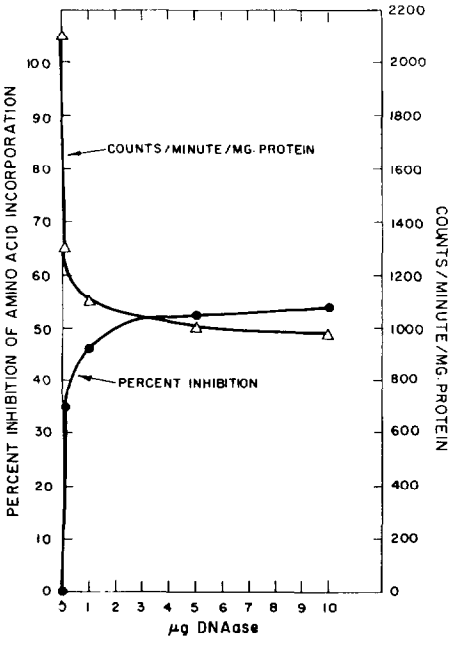

FIG. 7.—The effect of increasing concentrations of DNAase upon C^{14}-L-valine incorporation into protein. △ Counts/min/mg protein, ● Percent inhibition of amino acid incorporation. The composition of the reaction mixtures is presented in Table 1. 2.1 mg 30,000 × *g* supernatant solution protein (S-30) were present in each reaction mixture. Samples were incubated at 35° for 60 min.

the incorporation was more rapid during the first 30 min of incubation than during the next 60 min. At the end of 90 min of incubation, however, incorporation had not stopped. Addition of 10 μg crystalline DNAase/ml of reaction mixture did not affect the initial rate of incorporation, but incorporation ceased after 30 min. These results demonstrate that reaction mixtures must be incubated for more than 20 min to obtain reproducibly the DNAase effect.

The sensitivity of the system to DNAase is presented in Figure 7. When 0.1 μg. DNAase were added, approximately 70 per cent of the maximum DNAase inhibition was obtained. An inhibition that was almost maximal was obtained with 1.0 μg DNAase/ml. Increasing the concentration of DNAase 10-fold beyond this did not appreciably increase the inhibitory effect of DNAase, thus negating the presence of a contaminant inhibitor in the crystalline DNAase preparation. Contamination of DNAase with traces of proteolytic enzymes and RNAase seemed likely, since commercial DNAase is prepared from pancreas. Therefore, different preparations of crystalline DNAase were tested for both proteolytic and RNAase activity. Their purity varied widely. Crystalline DNAase obtained from the Worthington Biochemical Company (Lot No. D692-95-7) was the purest preparation tested and contained less than 0.3 per cent by weight of material which had a proteolytic enzyme activity and less than 0.001 per cent RNAase activity, corresponding to less than 0.05 μg trypsin and 0.0001 μg RNAase per 10 μg DNAase. In Table 2, the effects of these concentrations of RNAase and trypsin, singly and combined, upon C^{14}-valine incorporation into protein are presented. The system

TABLE 2

THE EFFECT OF RNAASE AND TRYPSIN UPON C^{14}-L-VALINE INCORPORATION INTO PROTEIN

Experiment no.	Additions	Counts/min/mg protein
1	Complete	374
	" + 0.0001 μg RNAase	352
	" + 0.001 μg RNAase	206
	" + 0.01 μg RNAase	69
	" + 0.1 μg RNAase	18
	" + 1.0 μg RNAase	9
	" Deproteinized at zero time	8
2	Complete	374
	" + 10 μg DNAase	234
	" + 0.05 μg Trypsin	394
	" + 0.05 μg Trypsin + 0.0001 μg RNAase	402
	" Deproteinized at zero time	8

The composition of the reaction mixtures is presented in Table 1. 2.8 mg S-30 protein were added to each reaction mixture. Samples were incubated at 35° for 60 min.

TABLE 3

THE EFFECT OF POLYANIONS UPON THE DNAASE-INHIBITED INCORPORATION OF C^{14}-L-VALINE INTO PROTEIN AND THE EFFECT OF A DNAASE DIGEST OF DNA UPON INCORPORATION

Experiment no	Additions	Counts/min/mg protein
1	Complete	2,040
	" + 10 μg DNAase	825
	" + " " + 100 μg polyadenylic acid	685
	" + " " + 100 μg polyglucose carboxyl derivative	810
	" + 100 μg polyadenylic acid	2,430
	" + 100 μg polyglucose carboxyl derivative	2,150
	" Deproteinized at zero time	7
2	Complete	1,842
	" + 100 μg DNAase digest of salmon sperm DNA	1,825
3	Complete	604
	" + 10 μg DNAase + 1.0 ml reaction mixture incubated with 10 μg DNAase for 60 minutes	661

The components of the reaction mixtures and the incubation conditions are presented in Table 1. 2.0 and 1.0 mg W-Rib and S-100 protein were present in Experiments 1 and 2. In Experiment 3, 2.1 mg S-30 protein was present in complete systems. 2.1 mg S-30 protein was also present in reaction mixture incubated with DNAase for 60 min. Samples were incubated at 35° for 20 min.

was extremely sensitive to RNAase. As little as 0.001 μg RNAase/ml of final reaction mixture depressed amino acid incorporation. The addition of 0.05 μg of trypsin and 0.0001 μg RNAase (the amount of trypsin and RNAase in 10 μg of the DNAase preparation used) had no effect upon the incorporation. This DNAase preparation was used for all subsequent work with DNAase. It should be emphasized that crystalline DNAase obtained from commercial sources varies widely in its content of trypsin and RNAase and thus should be assayed before use.

The data of Table 3 demonstrate that addition of polyanions such as polyadenylic acid and a polymer of glucose carboxylic acid did not reverse the inhibition obtained upon addition of DNAase. Higher concentrations of polyanions than those shown in Table 3 were inhibitory. Addition of a DNAase digest of highly polymerized salmon sperm DNA had no effect upon amino acid incorporation. In Experiment 3, Table 3, an additional experiment of this sort is presented. A reaction mixture containing 2.1 mg S-30 protein was incubated with 10 μg DNAase for 60 min. During this period the DNA contained in the enzyme would have been largely digested. Also, after 60 min, amino acid incorporation into protein had completely ceased. This reaction mixture, containing the endogenous, di-

gested DNA products, was then added to a fresh reaction mixture to see whether products of DNAase digestion were inhibitory. The results show that the products of DNAase digestion were not inhibitory.

Discussion.—A considerable amount of evidence has been obtained indicating that C^{14}-valine incorporation into protein in this system was not due to the presence of contaminating intact *E. coli* cells. The extracts were repeatedly centrifuged at 30,000 × *g*, and the pellet containing intact cells and debris was discarded. No intact cells or protoplast-like bodies were microscopically visible in the supernatant fluid. Neither ribosomes nor 105,000 × *g* supernatant solution alone incorporated appreciable quantities of C^{14}-valine. Both fractions were needed. In addition, combined extracts were almost totally inactive if ATP and an ATP-generating system were omitted.

The rate of amino acid incorporation proceeded rapidly for approximately 30 min and then gradually decreased. The incorporation had many characteristics expected of *de novo* protein synthesis. It required ATP and an ATP-generating system, was stimulated by a mixture of other L-amino acids, and was strongly inhibited by low concentrations of puromycin, chloramphenicol, and RNAase. In addition, the incorporation could be partially inhibited by addition of DNAase. The initial rate of incorporation was not inhibited by DNAase, in contrast to the extremely sensitive inhibition of the portion of the incorporation occurring after 20 min of incubation. As low as 0.1 μg DNAase per ml of reaction mixture greatly inhibited the incorporation occurring after 20 min of incubation. Various commercial preparations of crystalline DNAase were assayed for contamination with proteolytic enzyme activity and RNAase. Some were heavily contaminated. The maximum amount of trypsin and RNAase present as contaminants in the crystalline DNAase preparation used in this study had little effect upon C^{14}-valine incorporation when added to the system. Furthermore, if a trace contaminant in the DNAase were responsible for inhibiting amino acid incorporation, a correspondingly greater inhibition of amino acid incorporation would be expected when high concentrations of DNAase were used. The data of Figure 7 demonstrate that almost maximal inhibition was obtained with 1 μg DNAase per ml of reaction mixture. Increasing the DNAase concentration 10-fold did not appreciably increase the inhibition.

The data of Table 3 demonstrate that the products of DNAase digestion were not inhibitory in this system and that polyanions cannot non-specifically reverse a DNAase-inhibited incorporation system, in contrast to reports of such non-specific reversal in thymus nuclei.[9]

Although the mechanism of synthesis of template or "messenger" RNA has remained an enigma, enzymes possibly involved in this process are being studied.[10–12] It is not possible to say whether intact DNA is necessary for amino acid incorporation into protein in the later stages of incubation in this system. One possibility, however, which is consonant with all of the known facts is that the initial rate of amino acid incorporation is primarily due to the completion of partially finished peptides linked to RNA templates. If template RNA were used only once, amino acid incorporation would cease as soon as the peptide chains were finished. Inhibition by DNAase observed in this cell-free system may be due to the destruction of DNA and its resultant inability to serve as templates for the synthesis of

template RNA. Other explanations, however, are fully plausible, and it is not possible at this state to rule out alternative interpretations. In the following paper, further experiments on amino acid incorporation using the system described here are presented. It will be shown that in addition to the usual requirements, the system is stimulated by template RNA.

Summary.—Cell-free *E. coli* extracts have been obtained which actively incorporate amino acids into protein. Methods were devised whereby these extracts could be dialyzed and stored for long periods of time at $-15°$ without undue loss of activity. The characteristics of amino acid incorporation by such stored extracts were strongly suggestive of *de novo* protein synthesis, for incorporation required both ribosomes and 105,000 $\times$ *g* supernatant fractions, ATP and an ATP-generating system, was stimulated by a mixture of other L-amino acids, and was markedly inhibited by puromycin, chloramphenicol, and RNAase. The initial rate of amino acid incorporation was not inhibited by DNAase; subsequent incorporation was greatly inhibited. The possible relationship of the DNAase inhibition of amino acid incorporation into protein to the synthesis of "messenger" RNA was briefly discussed.

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