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*CHARACTERIZATION OF A RIBOSOME-LINKED GUANOSINE TRIPHOSPHATASE IN ESCHERICHIA COLI EXTRACTS**

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Communicated by Fritz Lipmann, October 28, 1964

The present study follows up a briefly reported observation by Monro[1-3] of a specific hydrolysis of GTP catalyzed by combination of a supernatant fraction with ribosomes. The following is an attempt to correlate more closely the GTPase effect with amino acid polymerization using as a test system the polyuridylic acid-mediated polyphenylalanine synthesis in *E. coli*.[4]

Methodology.—Synthesis of GTP-γ-P^{32}: Photophosphorylation with crude spinach chloroplast preparations, very low in myokinase, was used to make GTP-γ-P^{32} from GDP and P^{32}-phosphate. The chloroplasts were isolated essentially as described by Jagendorf and Avron.[5] The reaction mixture contained in a 3-ml volume: 200 μmoles of Tris-HCl pH 7.8, 25 μmoles of $MgCl_2$, 0.1 μmole of phenazine methosulfate, 1 μmole of GSH, 8 μmoles of GDP, 10 μmoles of potassium phosphate pH 7.8, 0.2 ml of the chloroplast suspension, and about 1 mc of neutralized P^{32}-phosphate which had been purified by passage through a small column (0.5 cm $\times$ 1 cm) of the acid form of Dowex-50. The suspension was illuminated for 15 min at 15–20° in a glass water bath $1\frac{1}{2}$ inches above a General Electric 500-w photoflood lamp, and the reaction terminated by boiling for 3 min.

The radioactive GTP was isolated on a 2-ml Dowex-1 (HCO_3) column (0.5 cm I.D.) at 4°. The column was washed with 200 ml of 0.4 *M* $KHCO_3$ to remove unreacted inorganic phosphate, and the GTP^{32} was eluted with 0.8 *M* $KHCO_3$. The radioactive fractions were pooled and treated

with $1^1/_2$ equivalents of acid Dowex-50, the resin was filtered off, and the solution neutralized to pH 7.8 and reduced to 3–5 ml by lyophilization.

Assay for GTPase and polyphenylalanine synthesis: Unless indicated, both GTPase and incorporation were measured in the same reaction mixture. Composition of the assays are given in the legends of each table and figure. The reaction was terminated by adding 0.5 ml of a 0.02 *M* solution of silicotungstic acid in 0.02 *N* H_2SO_4;[6] 1.25 ml of 0.001 *M* potassium phosphate pH 6.8 was added as carrier, and the tubes were centrifuged for 5 min at about 2,000 × *g*. The supernatant was carefully decanted for the estimation of phosphate. The pellet was resuspended in 2.5 ml of 5% trichloroacetic acid (TCA), heated for 10 min at 90°, and filtered on a nitrocellulose membrane filter. The membrane was washed with four portions of 2.5 ml of 5% TCA 0.1 *M* in carrier phosphate, fixed to a flat planchet, dried, and counted. This measured the C^{14}-phenylalanine incorporated into hot TCA-insoluble material; no detectable amounts of P^{32} were incorporated into either hot or cold TCA-insoluble material. To measure GTPase activity in the supernatant, 0.5 ml of a 5% solution of ammonium molybdate in 4 *N* H_2SO_4 was added. The phosphomolybdate complex was extracted into 2.5 ml of iso-butanol: benzene (1:1 v/v) according to Martin and Doty,[6] and a 2-ml aliquot of the organic phase was evaporated and counted. The radioactive inorganic phosphate determined by this method was taken as a measure of enzyme activity when corrected by controls lacking both enzymes and ribosomes. Procedures for sucrose density gradient centrifugation have been described,[7] as has the procedure for the preparation of C^{14}-phenylalanyl-sRNA.[8]

Preparation of supernatant fraction, transfer enzymes, and ribosomes: Separation of the nonparticulate fraction from the ribosomes of *E. coli* B log cells was according to a slightly modified procedure of Wood and Berg.[9] Cells and debris were removed by centrifugation at 32,000 × *g* for 30 min. To the supernatant fluid was added 1.4 gm of $(NH_4)_2SO_4$ per 10 ml of solution; pH was kept at 6.5–7.5 during this operation. The precipitate that formed was removed at 32,000 × *g* for 40 min. The clear supernatant was centrifuged for 3 hr at 105,000 × *g* to remove ribosomes. The upper $^3/_4$ of the supernatant solution was removed and dialyzed overnight against 3 liters of 0.01 *M* Tris-HCl pH 7.4, 0.01 *M* $MgCl_2$, and 0.001 *M* 2-mercaptoethanol. The clear ribosomal pellets were covered with 0.01 *M* Tris-HCl pH 7.4, 0.01 *M* $MgCl_2$ buffer, and allowed to soften overnight at 0°. Ribosomes were resuspended by gentle stirring, centrifuged at 15,000 × *g* for 15 min to remove aggregated material, and sedimented as before; this washing procedure was repeated three times. Finally, the ribosomes were taken up in a small volume of the same buffer to give a solution that was at least 40 mg per ml. One mg dry weight of ribosomes was assumed to be equivalent to 14.4 O.D. units at 260 mμ.

The dialyzed supernatant solution was treated with 1% protamine sulfate as has been described.[9] The protamine supernatant was fractionated with ammonium sulfate in the manner described for a similar streptomycin sulfate supernatant.[2] The active fraction contained about 26 mg of protein per ml and was designated as the 65% AS fraction; it is similar to the ammonium sulfate fractions previously described,[2, 10] in that it contained both the A and the B transfer fractions.[2] For further fractionation, 84 mg of a 3 ml calcium phosphate slurry,[11] washed three times with 10 ml of 0.001 *M* Tris-HCl pH 7.4, were mixed with 2 ml of 65% AS fraction (54 mg of protein); 10 ml of 0.001 *M* Tris pH 7.4 were added, and the mixture was stirred for 15 min at 0°. The gel was washed three times with 10-ml portions of 0.001 *M* Tris pH 7.4 and once with 0.33 *M* NH_4Cl pH 7.4. An active fraction that contained both the A and B enzyme activities was eluted from the gel with 10 ml of 0.33 *M* $(NH_4)_2SO_4$ pH 8.2.

Nucleoside triphosphates were obtained from Pabst Laboratories, polyuridylic acid (poly U) potassium salt from Miles Chemical Company, and phenazine methosulfate from Sigma Chemical Company. L-phenylalanine-U-C^{14} was purchased from Schwarz BioResearch, Inc., and P^{32}-phosphate from Oak Ridge National Laboratory.

The Products of GTP Hydrolysis.—It has been shown that GTP is hydrolyzed to GDP and phosphate by both the *E. coli*[1] and the rabbit reticulocyte systems.[12] We have identified GDP as a product of hydrolysis by electrophoresis or chromatography of the reaction mixture, with C^{14}-GTP as substrate. Two spots corresponding to GDP and GTP markers could be demonstrated in three different ways: by radioautography, by UV quenching at neutral pH, and by fluorescence at acid pH; but no GMP appeared.

Comparison of Phenylalanine Incorporation with GTP Hydrolysis.—To compare GTP hydrolysis and phenylalanine polymerization, the two reactions were measured under the conditions optimal for polyphenylalanine synthesis from phenylalanyl sRNA, namely, 10 mM Mg^{++} and pH 7.4, although for GTP hydrolysis 25 mM Mg^{++} and pH 9 are optimal. The complementarity for GTP hydrolysis of a supernatant fraction and washed ribosomes is illustrated in Table 1. It appears

TABLE 1

COMPARISON OF GTPASE AND PHENYLALANINE INCORPORATION

Conditions	GTPase (cpm)	Incorporation (cpm)
Complete	1042*	340†
- enzyme	35	38
- ribosomes	87	21
- C^{14}-phe-sRNA	421	21
- poly U	496	34

* 6.26 mμmoles of GTP.
† 8.27 μμmoles of phenylalanine.
The reaction mixtures were preincubated for 5 min at 30° in the absence of GTP and soluble enzymes. They were chilled to 0°, GTP and the soluble enzymes were added, and the incubation was continued for 5 min at 0°. The complete system contained: 12.5 μmoles of Tris-HCl pH 7.4; 3 μmoles of 2-mercaptoethanol; 40 μmoles of NH_4Cl; 2 μmoles of $MgCl_2$; 5 μg of poly U; 0.123 mg of C^{14}-phenylalanyl sRNA, specific activity 72 μC per μmole, 1,468 cpm; 0.129 mg of 3-times washed ribosomes; 50 μmoles of GTP-γ-P^{32}, 10,427 cpm; and 25.9 μg of a soluble enzyme fraction eluted from calcium phosphate as described in *Methodology*. The final volume was made up to 0.25 ml with water. The reaction was terminated and the P^{32}-phosphate removed from the C^{14}-labeled materials as described in *Methodology*.

TABLE 2

MONOVALENT CATION REQUIREMENTS FOR GTPASE ACTION AND POLYPHENYLALANINE SYNTHESIS

Monovalent salt	GTPase (cpm)	Incorporation (cpm)
None added	1470	22
NH_4Cl	2712	633
	3251*	549*
NaCl	165	11
LiCl	318	43

* 0.08 *M* NH_4Cl.
The assay mixture contained the following in 0.25 ml: 12.5 μmoles of Tris-HCl pH 7.4; 2.5 μmoles of $MgCl_2$; 3 μmoles of 2-mercaptoethanol; 0.494 mg of 3-times washed ribosomes; 5 μg of poly U; 25 μg of a 65% AS fraction (dialyzed) of the transfer enzymes; 0.05 μmole of GTP^{32}, 9400 cpm; and 0.123 mg of C^{14}-phenylalanyl sRNA, specific activity 72 μC/μmole, 1468 cpm. Monovalent salt was added to a final concentration of 0.16 *M* and enough water to make a constant final volume. Incubation was for 10 min at 30°.

that GTPase is stimulated by poly U and phenylalanyl sRNA, the activity being lowest when both poly U and phenylalanyl sRNA are absent. Amino acid-free sRNA, prepared according to von Ehrenstein and Lipmann,[13] can substitute for sRNA charged with phenylalanine in stimulating the GTPase, and periodate-treated sRNA is also stimulatory.[14] Control experiments have excluded the stimulation of phosphate liberation from GTP by these nucleic acid preparations to be due to a phosphorolytic incorporation of guanylic acid into cold TCA-insoluble material. The possibility that the addition of sRNA and poly U might indirectly influence the rate of hydrolysis by supplying bound Mg^{++} to the system appears unlikely since there was no sparing effect on the Mg^{++} requirement at low concentrations.

Incorporation of phenylalanine from phenylalanyl sRNA in this system has been shown to be dependent upon the presence of either potassium or ammonium ions.[8, 15] The GTPase may be stimulated by NH_4Cl (Table 2) but, unlike the polymerizing activity, is not dependent upon added NH_4^+, the optimum for GTPase being about 0.08 *M* or half as high as for incorporation. It seems significant, however, that sodium or lithium ions known to inhibit polymerization of phenylalanine[15] also inhibit GTPase (Table 2).

A strong inhibition by GDP of both amino acid incorporation and GTPase is shown in Figure 1; the apparent Michaelis constant for the incorporation is considerably smaller (1.6×10^{-5} *M*) than that for the GTPase (1.8×10^{-4} *M*). Furthermore, the plot shows GDP to inhibit both reactions competitively. At-

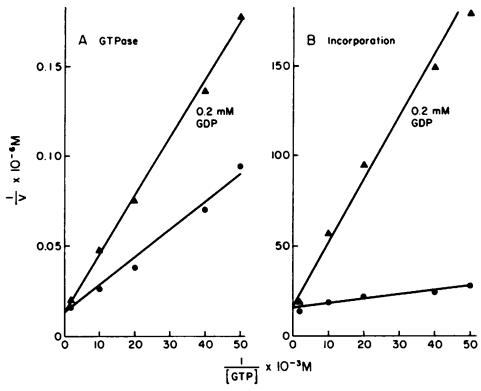

FIG. 1.—GDP inhibition of GTPase and amino acid incorporation. Reaction mixtures contained in 0.25 ml: 12.5 μmoles of Tris-HCl pH 7.4; 3 μmoles of 2-mercaptoethanol; 40 μmoles of NH_4Cl; 2.5 μmoles of $MgCl_2$; 50 μg of a soluble enzyme prepared according to Nathans and Lipmann;[10] 0.3 mg of washed ribosomes; GTP-γ-P^{32} as indicated, 1 mμmole equaled 142 cpm; 5 μg of poly U; and 0.123 mg of C^{14}-phenylalanyl sRNA, specific activity 72 μC/μmole, 1468 cpm. Where indicated, GDP was added to a final concentration of $0.2 \times 10^{-3} M$. Incubation was for 10 min at 25°C. Both GTPase activity and polyphenylalanine synthesis were determined in the same tube, as described in *Methodology*. Velocity is expressed as moles of GTP hydrolyzed, or phenylalanine polymerized, per liter per 10 min.

tempts to look for a ribosome-dependent exchange between GTP and GDP or GTP and phosphate have been frustrated so far by too high blanks in the supernatant fraction; here, meaningful experiments demand further purification.

Nucleoside Triphosphate Specificity.—Table 3 shows that GTP is rather specific for

TABLE 3

NUCLEOSIDE TRIPHOSPHATE SPECIFICITY FOR POLYPHENYLALANINE SYNTHESIS

Nucleoside triphosphate	C^{14}-phenylalanine transferred (cpm)
None	6
GTP	433
ITP	185
ATP	27
UTP	14
CTP	7

Reaction mixtures were the same as given in Table 1 except that unlabeled nucleoside triphosphates were used at a final concentration of 0.1 μmole per ml. Incubation was for 7 min at 30°.

TABLE 4

RIBOSOME STIMULATION OF NUCLEOSIDE TRIPHOSPHATE HYDROLYSIS

Nucleoside triphosphate	mμMoles of γ-P^{32}-phosphate released— Ribosomes	Enzyme	Ribosomes + enzymes
GTP^{32}	0.32	0.57	9.53
ATP^{32}	0.12	1.29	1.95
ITP^{32}	0	1.65	1.68
CTP^{32}	0.11	0.84	0.31
UTP^{32}	0	0.12	0.13

Reaction mixtures contained: 12.5 μmoles of Tris-HCl pH 7.4, 2.5 μmoles of $MgCl_2$, 3 μmoles of 2-mercaptoethanol, 40 μmoles of NH_4Cl, 0.494 mg of 3-times washed ribosomes, 25 μg of a 65% AS fraction (dialyzed) of the transfer enzymes, 0.05 μmole of the indicated nucleoside triphosphates (GTP^{32}, 7,630 cpm; ATP^{32}, 10,688 cpm; ITP^{32}, 13,289 cpm; UTP^{32}, 13,253 cpm; CTP^{32}, 2,865 cpm), and enough water to make the final volume 0.25 ml. Incubation was at 30° for 7 min.

polyphenylalanine synthesis although ITP has some activity. To demonstrate GTP specificity for incorporation, a limiting concentration of soluble enzyme must be used, probably because of contaminating kinases capable of phosphorylating traces of guanosine nucleotides contaminating the tested nucleoside triphosphates. Arlinghaus *et al.*[12] and Fessenden and Moldave[16] have found a high degree of specificity for GTP in reticulocyte and rat liver systems, respectively. Table 4 illustrates the specificity of GTP for the ribosome-linked GTPase, since only GTP shows the specific increase in breakdown when washed ribosomes are added to the soluble enzyme.

Ribosome Participation.—Figure 2 shows the results of an experiment designed to determine what part of the ribosome population can stimulate the GTPase. Ribosomes washed three times were first charged with poly U at 0° in a solution containing 0.01 *M* $MgCl_2$, 0.01 *M* Tris-HCl pH 7.4.[7] They were then centrifuged through a gradient containing 0.16 *M* NH_4Cl, 0.01 *M* $MgCl_2$, and 0.01 *M* Tris-HCl pH 7.4; fractions were collected and assayed for both phenylalanine incorporation

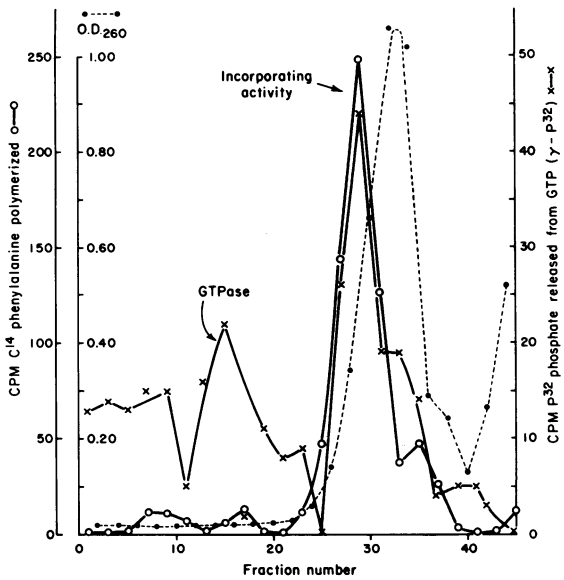

FIG. 2.—Assay of gradient fractions for polyphenylalanine synthesis and GTPase activity. The mixture contained: 12.5 μmoles of Tris-HCl pH 7.4, 2.5 μmoles of $MgCl_2$, 3 μmoles of 2-mercaptoethanol, 75 μg of potassium poly U, and 1 mg of 3-times washed ribosomes, all kept at 0° and in a volume of 0.25 ml. A sucrose density gradient containing 10^{-2} *M* $MgCl_2$, 10^{-2} *M* Tris-HCl pH 7.4, and 0.16 *M* NH_4Cl, was prepared as described;[7] 0.2 ml of the ribosomal mixture was placed on the gradient. Sedimentation analysis was conducted at 10° for 1 1/4 hr at 37,000 rpm in the SW-39 rotor of the Spinco model L centrifuge. Fractions, each consisting of 2 drops, were collected at 0°; alternate samples were used for the optical density measurement at 260 mμ and for the enzyme assays. Enzyme reaction mixtures were similar to Table 1 with the following exceptions: the entire sucrose density gradient fraction replaced the ribosomes; the final $MgCl_2$ concentration was 10 mM, accounting for the magnesium added with the gradient fractions; 50 mμmoles of GTP-γP^{32}, 3040 cpm, and 32.4 μg of a dialyzed 65% AS fraction were used. Incubation was for 7 min at 30°. The GTPase and incorporating activity were determined as outlined in *Methodology*. At the peak tube, no. 29, 724 $\mu\mu$moles of GTP were hydrolyzed and 6.4 $\mu\mu$moles of C^{14}-phenylalanine were polymerized.

and GTPase. Although considerable GTP hydrolysis is associated with the heavier ribosomes shown at the left of the diagram, the major peak overlaps nicely with the main peak of incorporating activity that appears on the fraction just preceding the 70S peak. Thus, the same portion that is most active for polyphenylalanine synthesis is also the most active for GTPase.

To check if only one of the separable ribosomal units might be sufficient for GTPase activation, washed ribosomes were separated into subunits as described in

TABLE 5

EFFECT OF SEPARATION OF RIBOSOME SUBUNITS ON GTPASE AND INCORPORATION

No.	Ribosome preparation	GTPase (sp. act.)*	Incorporation (sp. act.)*	Ratio GTPase: incorp.
1.	70S	7500	43.8	171
2.	Separated 50S or 30S	280	—	—
3.	Reconstituted 50S and 30S	1950	13.4	145

* $\mu\mu$moles/mg of ribosomes/min.

Washed ribosomes were dialyzed overnight against 100 vol of 0.01 *M* Tris-HCl pH 7.4, and 6 mg placed on a 5-20% sucrose density gradient. Centrifugation was for 4 hr at 25,000 rpm in the SW-25 rotor. Samples were taken as described by Gilbert.[17] Assay of the gradient fractions with added 65% ammonium sulfate enzyme showed very little GTPase activity. The 50S and 30S fractions were combined, dialyzed for 3 hr against 10^{-2} *M* $MgCl_2$, 10^{-2} *M* Tris pH 7.4, and assayed for GTPase and incorporating activities as described in *Methodology*. Incubation was for 7 min at 30°. Data reported as specific activity in respect to the ribosomes.

Table 5. The table indicates that the separated fractions lost most activity, whereas when allowed to reassociate, GTPase activity was restored. Unfortunately, our washed ribosomes had a diminished activity on recombination, but as shown in no. 3, incorporation and GTPase diminished in equal proportion.

Effect of Heat on GTPase.—A partially purified supernatant fraction[10] containing both of the complementary A and B fractions,[2] was heated and assayed for GTPase and for incorporating activity. It can be seen in Figure 3 that heating to 55° destroys 75 per cent of the incorporating activity while GTPase actually increases. It appears, therefore, that short heating destroys the effect on protein synthesis but stimulates GTPase, which disappears first on exposure to 10–15° higher temperature. This could be interpreted as an uncoupling, meaning that the GTP hydrolysis may be due to a partially denatured enzyme that degenerates from energy transfer to hydrolytic energy dissipation. It is relevant that the relatively unstable B complement for incorporation also carried most of the GTPase.[2] Incorporation could be restored to two thirds of that before heating by addition of a sample of separated B fraction.

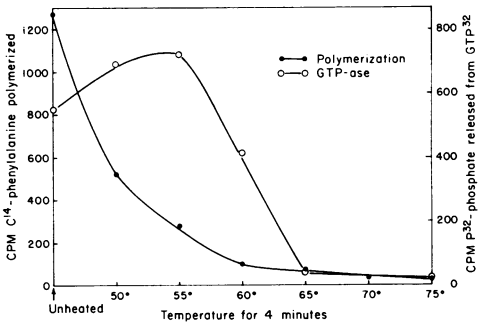

FIG. 3.—Differential heat inactivation of transfer and GTPase activities. The DEAE-cellulose enzyme fraction, prepared according to Nathans and Lipmann,[10] was heated for 4 min at the temperatures indicated. Transfer and GTPase activities were measured in a volume of 0.25 ml in the following mixture: 50 mM Tris-HCl pH 7.4; 10 mM PEP; 7.5 μg of pyruvate kinase; 12 mM $MgCl_2$; 30 mM KCl; 12 mM GSH; 2 μg of poly U; 0.878 mg of 4-times washed ribosomes; 1 mM GTP^{32}, 4370 cpm per tube; 0.073 mg of C^{14}-phenylalanyl sRNA, 2500 cpm per tube, specific activity 45 μC/μmole; and 30 μg of DEAE-cellulose fractionated enzyme treated as described above. Incubation was at 30° for 10 min.

Test of Antibiotics.—Several antibiotics known to inhibit protein synthesis at some stage between aminoacyl sRNA and polypeptide formation were assayed for effect on GTPase. Chloramphenicol, 80 μg per ml, puromycin 4×10^{-4} *M*, neomycin, 80 μg per ml, kanamycin, 20 μg per ml, streptomycin, 70 μg per ml, and tetracycline, 10 μg per ml, were tested. None were found to inhibit GTPase at concentrations known to inhibit polymerization.

Comments and Summary.—The close overlap of amino acid incorporation and GTPase activity with the poly U-ribosome fraction in the gradient centrifugation profile of Figure 2 is most suggestive that a GTP split is connected with protein synthesis. It is difficult to consider this as accidental. Furthermore, a GDP inhibition of both GTPase and, even more strongly, of polypeptide synthesis, favors connecting the two reactions. Nevertheless, a relatively weak response to change in ionic development, a response to, but not a dependence on, sRNA makes the parallel incomplete. One of the most interesting effects is the destruction of the polymerization function by heating to 55° which, on the other hand, leads to a stimulation of GTP hydrolysis. We are inclined to interpret this as an uncoupling of a functional transfer of the terminal phosphoryl of GTP.

One has strenuously looked for a use of energy in one or the other phase of the polymerization process. Although the group potential of the amino acid in the

aminoacyl sRNA should be high enough to cover peptide synthesis, it is mostly the energy of the peptidyl rather than the aminoacyl link to sRNA that is invested in the formation of new peptide bonds. But by removing the influence of the amino group from the ester link, the group potential of peptidyl sRNA may be substantially lower than that of aminoacyl sRNA, as indicated by the decrease in sensitivity to alkali.[17, 18] Therefore, there is the possibility that an additional activation may be advantageous.

One may postulate quite a different energy requirement for a presumed forward move of messenger RNA on the ribosomes parallel to the extending peptide chain. It is an attractive proposition, already suggested by Watson,[19] that after every peptide bonding to a new aminoacyl sRNA, the lengthened peptidyl sRNA slides from acceptor site to donor site and, at the same time, pulls the messenger RNA one notch ahead. This movement is repeated every time a peptide bond is formed; it could be coincidental with an alternate contraction and expansion of the ribosome dependent on GTP split. This may be related to recently discussed effects of GTP on ribosomal or accessory proteins suggested by Hoagland *et al.*[20] It seems significant, in this respect, that the GTPase function requires the assembly of the 50S and 30S particles into the whole ribosome, this possibly indicating interaction between the aminoacyl sRNA on the 50S,[21] and messenger RNA on the 30S parts.[22]

Arlinghaus *et al.*[12, 23] have reported on the association of GTPase with one of their separable aminoacyl sRNA transfer enzymes from rabbit reticulocytes. This fraction + GTP promotes a poly U-dependent binding of phenylalanyl sRNA to ribosomes. They have interpreted the extensive hydrolysis of GTP to be due to the constant synthesis, breakdown, and resynthesis of an extremely labile intermediate. In the *E. coli* system, the likelihood, mentioned elsewhere,[3, 24] of an enzymatic nature of the binding may indicate a residue of supernatant on our washed ribosome. Here, however, no need of added GTP for binding of aminoacyl sRNA could be demonstrated.[24]

In conclusion, the quite close interrelationship between GTP split and amino acid polymerization favors, but far from proves, that a phosphoryl split is connected with some phase in protein synthesis.

* This work was supported in part by a grant from the National Science Foundation.

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*GUANIDINATED CYTOCHROME C**

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Communicated by Joseph S. Fruton, October 28, 1964

Nineteen of the 24 basic amino acid residues of horse heart cytochrome *c* are lysine residues. Their positive charge is thought to be of major consequence in maintenance of the native conformation of the molecule,[1] and is a factor also in the binding of cytochrome *c* to cytochrome oxidase,[2–4] and, perhaps, other components of the electron transfer chain. Partial acetylation, carbamylation, or succinylation of the protein leads to the loss of its activity in the cytochrome oxidase and succinate oxidase systems.[5–7] Partial guanidination, on the other hand, yields products which are active.[7, 8]

The work with partially guanidinated material was done with samples in which up to 13–17 of the lysine residues had undergone conversion. If, as has been suggested,[6, 9] one of the coordination positions about the heme iron of cytochrome *c* is occupied by a lysine residue, then this residue might be more refractory to modification than its noncoordinated counterparts, and guanidination of all 19 ϵ-amino groups, if attainable, would probably result in a product significantly different from that obtained upon partial reaction.

The studies reported in the present communication show that complete guanidination of horse heart cytochrome *c* can readily be accomplished. The fully guanidinated product closely resembles unmodified cytochrome *c* in a number of important properties, and remains active in the succinate and cytochrome oxidase systems.

Experimental.—Materials: Horse heart cytochrome *c* was obtained from Sigma Chemical Co. (Type III, iron content 0.43%). A sample of crystalline horse heart cytochrome *c*, the gift of Dr. E. Margoliash, was used in a few comparative measurements. *O*-Methylisourea hydrochloride was synthesized as described by Kurzer and Lawson.[10] It melted with decomposition at 122°. Analysis for Cl: calculated, 32.07; found, 32.00. α-Chymotrypsin was a 3-times crystallized preparation obtained from Worthington Biochemical Corp. It had a trypsinlike activity 0.01% that of pure trypsin, as measured by the hydrolysis of tosyl-L-arginine methyl ester.[11]

Guanidination of cytochrome c: All operations were performed at 2°. Cytochrome *c* (final concentration, 1%) was added to solutions of *O*-methylisourea (final concentration, 0.5 *M*) of pH 8.0–8.5, and the pH then adjusted to 11.0 with 6 *M* sodium hydroxide. The reaction mixtures were