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INHIBITION OF RETICULOCYTE RIBOSOMAL PROTEIN SYNTHESIS BY CHLORAMPHENICOL IN A CELL-FREE SYSTEM

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Abstract—In a cell-free system of rabbit reticulocyte ribosomes, native "messenger"-enriched ribonucleic acid-stimulated synthesis of protein is inhibited by chloramphenicol. Confirmation is provided for the previous observation that polyuridylic acid-stimulated incorporation of phenylalanine is inhibited by chloramphenicol. Under conditions of prolonged incubation, chloramphenicol may inhibit the synthesis of protein by ribosomal systems derived from either rabbit reticulocytes or bone marrow cells.

ALTHOUGH chloramphenicol is known to inhibit protein synthesis in bacteria[1–3] and in cell-free systems derived from certain species of bacteria[4, 5] no significant inhibition, has been demonstrated in cell-free systems of yeast[6] or, until recently, of mammalian cells.[7–9] The demonstration by Weisberger *et al.*[10] that polyuridylic acid-induced ribosomal protein synthesis in a cell-free system prepared from rabbit reticulocytes could be inhibited by chloramphenicol appeared to provide a new approach to the understanding of the mechanism of action of this drug. A recent report of Kucan and Lipmann,[11] however, cast doubt on both the validity and the significance of these observations.

In this report we have confirmed the work of Weisberger *et al.* and, in addition, have demonstrated that a native "messenger-enriched RNA" will stimulate the incorporation of labeled amino acids in a cell-free system derived from rabbit reticulocytes—a reaction that also is blocked by chloramphenicol.

Under the unique circumstance of prolonged (14-hr) incubation in a system with relatively small amounts of ribosomes, chloramphenicol will inhibit amino acid incorporation even in the absence of exogenous messenger RNA (m-RNA).

METHODS AND MATERIALS

Rabbit reticulocyte ribosomes were prepared by the method of Allen and Schweet[9] and, in the early experiments, centrifuged to a pellet only once. Later, ribosomes were spun down to a pellet, resuspended, and then recentrifuged. The second pellet was either resuspended in buffer and frozen in aliquots in test tubes, or the pellets were drained, washed, with buffer and frozen at −10° in Spinco tubes. The "pH 5-enzyme" was made by the technique of Keller and Zamecnik.[12] The RNA or total

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nucleic acid for stimulation was prepared by several techniques, e.g. that of Georgiev and Mant'eva,[13] Lashkov *et al.*,[14] or Kenny and Kull.[15] Both the incubation mixture and the wash procedure were initially patterned after that of Allen and Schweet,[9] but in later experiments the procedure of Weisberger *et al.*[10] was followed. In our latest work, the paper-disk wash technique of Mans and Novelli[16] was found to be simple and rapid. For protein analysis, either the technique of Lowry *et al.*[17] or Gornall *et al.*[18] was used. All radioactive samples were counted for 10 min in a Packard Tri-Carb liquid scintillation counter.

Commercially available materials were purchased: sodium guanosine 5′-triphosphate, phosphenol pyruvate (as the tricyclohexylammonium salt), and pyruvate kinase from Calbiochem (and C. F. Boehringer & Soehne); sodium salts of adenosine 5′-triphosphate, cytosine 5′-triphosphate, and uridine 5′-triphosphate from the Sigma Chemical Co.; DL-leucine-1-^{14}C (7·84 mc/mmole), DL-leucine-1-^{14}C HCl (17·1 mc/mmole), and DL-phenylalanine-3-^{14}C HCl (4·2 mc/mmole) from New England Nuclear Corp; L-leucine-U-^{14}C (20 mc/mmole), L-phenylalanine-U-^{14}C (140 mc/mmole), and L-valine-U-^{14}C (100 mc/mmole) from Schwarz BioResearch; polyuridylic acid potassium salt from Miles Chemical Co.; amino acids from Nutritional Biochemicals Corp; rat liver SRNA sodium salt from General Biochemicals; and Whatman 17 chromatography paper from Angell and Reeves, Inc. Chloramphenicol was kindly supplied by Dr. A. C. Bratton of Parke, Davis & Co. and potassium penicillin G by E. R. Squibb & Co.

RESULTS

Effect of chloramphenicol on the standard (1-hr incubation) reticulocyte system

It will be seen from Table 1 that chloramphenicol in a concentration of 0·5 μmole/ml

TABLE 1. INCORPORATION OF ^{14}C-AMINO ACIDS*

	Counts per min/mg ribosomal protein		
	Expt. 1	Expt. 2	Expt. 3
Complete incubation mixture	387	381	429
Complete incubation mixture	369	339	439
Complete incubation mixture	355	325	448
Complete incubation mixture	399	323	456
Incubation mixture minus ribosomes	10	35	6
Complete mixture plus 0·5 μmole chloramphenicol	406	322	399

* The incubation mixture, final volume 1·0 ml, contained 8·7 mg pH 5-enzyme protein, 2 μg iron ferrous ammonium sulfate, 20 μg pyruvate kinase, and the following, in μmoles: magnesium acetate, 10; potassium chloride, 50; pH 7·8 Tris–HCl, 100; 2-mercaptoethanol, 6; mixture of 18 amino acids, 0·05; GTP, UTP, CTP, 0·025; ATP, 1; phosphoenol pyruvate, 5; and DL-leucine-1-^{14}C (17·1 mc/mmole), 270,000 cpm per flask. Incubation was performed at 37° for 60 min.

failed to inhibit synthesis significantly (^{14}C-amino acid incorporation into TCA-insoluble material), as had been reported by Allen and Schweet.[9] The three experiments presented are characteristic of the findings of other experiments with varied concentrations of chloramphenicol and quantities of ribosomes or pH 5-enzyme.

Stimulation of amino acid incorporation by nucleic acid preincubation

To enhance the incorporation of labeled amino acids into protein, stimulation by a variety of nucleic acid preparations, as suggested by Fischer *et al.*[19] was attempted. DNA, total RNA, and a total nucleic acid extract were used with occasional success, but the response was not convincing. In all these experiments, any enhancement of incorporation remained unaffected by the chloramphenicol.

After the report of Weisberger *et al.*,[10] the importance of preincubating the complete reaction mixture, prior to the addition of the isotopically labeled compounds and the nucleic acid fractions, became evident. In Table 2 the result of such an

TABLE 2. STIMULATION BY NUCLEIC ACIDS OF INCORPORATION OF AMINO ACIDS AFTER A PREINCUBATION PERIOD OF 15 MIN*

	Counts/min/mg ribosomal protein
Control (no additions; average of duplicate determinations)	288
Rabbit liver RNA (6 mg) added	5,000
Rabbit liver (6 mg) plus 0·5 μmole CAP†	1,475
Rabbit liver DNA (1·0 mg)	840
Rabbit liver DNA (1·0 mg) plus 0·5 μmole CAP	940
Rabbit liver DNA (1·5 mg)	895
Rabbit liver DNA (1·5 mg) plus 0·5 μmole CAP	1,105

* The incubation mixture consisted of 1·0 ml containing 720 μg pH 5-enzyme protein, 200 μg ribosomal protein, 20 μg pyruvate kinase, and the following, in μmoles: magnesium chloride, 10; potassium chloride, 50; pH 7·8 Tris–HCl, 100; 2-mercaptoethanol, 6; mixture of 18 amino acids, 0·05; GTP, UTP, CTP, 0·025; ATP, 1; phosphenol pyruvate, 5; DL-leucine-1-^{14}C (17·1 mc/mmole), 270,000 cpm per flask. Preincubation 15 min. Incubation for 45 min at 37°.

† CAP = chloramphenicol.

enhancement after a 15-min preincubation is indicated. In this experiment, very large amounts of RNA and DNA were used. In later reaction mixtures, graded amounts of RNA were used to determine the dose–response relationship. The stimulatory properties of DNA were not explored further as they seemed to plateau

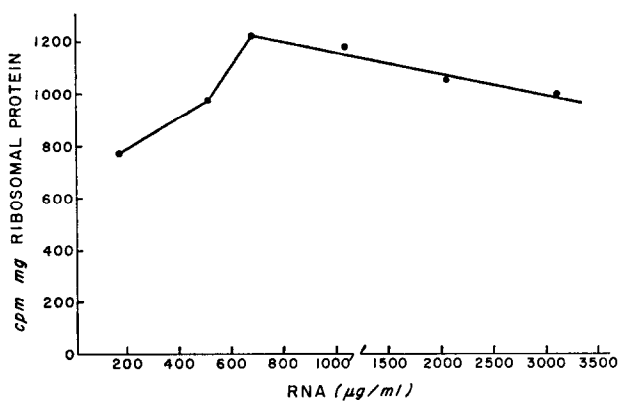

FIG. 1. Stimulation of incorporation of ^{14}C-leucine by RNA. The incubation mixture of 1·0 ml contained the same ingredients as those listed in Table 2; also, the periods of preincubation and incubation were the same. The stimulating RNA was derived from rabbit liver and was prepared by the technique of Lashkov *et al.*[14] Incubation at 37°.

at low levels and were unaffected by chloramphenicol. Although most preparations of messenger-enriched RNA caused stimulations that were approximately linear in the low dosage range, a plateau or inhibition-type curve was seen at higher dosages (Fig. 1). This paradoxical finding was repeated with several ribosome preparations, at least two different sources of stimulating RNA (rabbit bone marrow or liver), and three different preparatory techniques.

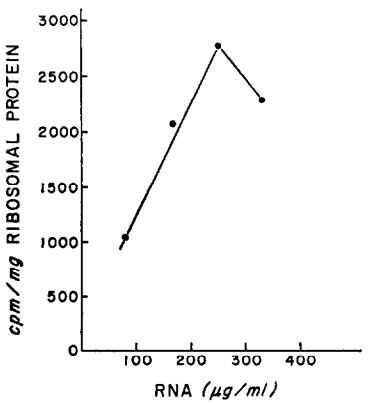

FIG. 2. RNA-stimulated ^{14}C-leucine incorporation. The incubation mixture consisted of 0·5 ml containing 364 μg pH 5-enzyme protein, 10 μg pyruvate kinase, 100 μg rabbit reticulocyte ribosomes, and the following, in μmoles: magnesium acetate, 5; potassium chloride, 25; pH 7·8 Tris-HCl, 50; 2-mercaptoethanol, 6; mixture of 18 amino acids, 0·05; GTP, CTP, UTP, 0·025; ATP 1; phosphoenol pyruvate 1·25; L-leucine-U-^{14}C (20 mc/mmole), 316,000 cpm per flask. The RNA was prepared from rabbit liver by the technique of Georgiev and Mant'eva.[13] Rat liver SRNA failed to stimulate the system. Preincubation, 45 min, incubation, 1 hr at 37°.

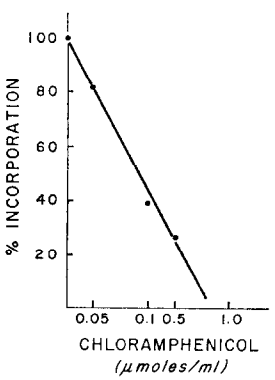

FIG. 3. Inhibition by chloramphenicol of the RNA-stimulated incorporation of ^{14}C-leucine. The incubation mixture the same as for Fig. 2.

Inhibition by chloramphenicol of the incorporation of amino acids stimulated by m-RNA

Having established the fact that native messenger-enriched RNA can stimulate the incorporation of amino acids by ribosomes, chloramphenicol was added to the system in several concentrations. In Fig. 2 each point represents the average of duplicate determinations with the zero-point based on the average of 18 control incubations, with the same reagents at the same time, but without m-RNA. In Fig. 3,

the inhibition by 1/2 log dilutions of chloramphenicol is depicted. Unfortunately the last point does not extrapolate to zero as one would expect but at this concentration (246 μg RNA), there is maximal stimulation, and it cannot be completely suppressed by the antibiotic.

Chloramphenicol inhibition of polyuridylic acid-stimulated synthesis of polyphenylalanine

The demonstration of this phenomenon by Weisberger *et al.*[10] has greatly encouraged further investigations into the mechanism of action of chloramphenicol. Since these findings were in agreement with our results that demonstrated stimulation by RNA, we assumed the report to be correct. Recently, however, Kucan and Lipmann[11] have raised doubts concerning the reproducibility of the inhibition of polyuridylic acid-stimulated synthesis of polyphenylalanine. Hence we have repeated the experiments of Weisberger *et al.* many times and have obtained very similar results. One such experiment is illustrated in Fig. 4, in which the enhancement of incorpora-

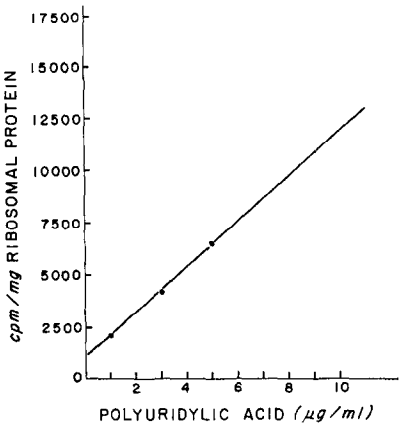

FIG. 4. Polyuridylic acid-stimulated incorporation of ^{14}C-phenylalanine. The incubation mixture was the same as for Fig. 2, except that L-phenylalanine-U-^{14}C (140 mc/mmole), 607,500 cpm per flask was used, and polyuridylic acid as indicated. Incubation time 1 hr (no preincubation).

tion by RNA is almost linear. In Fig. 5 the suppression of RNA stimulation by chloramphenicol is reproducible, although the responses are not quite linear.

Effect of prolonged incubation and low amounts of ribosomes and pH 5-enzyme on chloramphenicol inhibition

To determine whether the duration of exposure to chloramphenicol would influence protein synthesis, 14-hr (overnight) incubations at 37° in a Dubnoff shaker were performed with ribosomes obtained from either reticulocytes or bone marrow. As far as possible, all components of the system were prepared by aseptic technique or were sterile-filtered. To each incubation flask 600 μg penicillin-G was added to prevent bacterial growth secondary to any unavoidable contamination. At the end of the incubation period there was no obvious evidence of bacterial contamination. The control flasks and several other selected flasks were cultured on blood–agar

plates and these failed to show bacterial growth. As indicated in Table 3. some degree of inhibition was found in every case, whether the ^{14}C-amino acid was leucine or phenylalanine, and whether reticulocyte or bone marrow ribosomes were used. The low levels of ribosomes were deliberately selected to favor a manifestation of the effect of chloramphenicol. Although, as described, great care was exercised to exclude

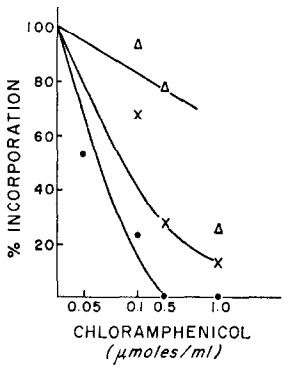

FIG. 5. Chloramphenicol inhibition of polyuridylic acid-stimulated incorporation of ^{14}C-phenylalanine. The incubation mixture was the same as for Fig. 2. Polyuridylic acid concentrations: 1 μg/ml ●—●; 3 μg/ml ×—×; 5 μg/ml △—△. Endogenous (control) values were subtracted from all the points plotted. The curve at the 5 μg/ml level is representative of our other experiments, and the inhibition at 1·0 μmole/ml is regarded as an experimental error.

TABLE 3. INCORPORATION OF ^{14}C-AMINO ACIDS DURING A 14-HR PERIOD OF INCUBATION*

	Per cent utilization, as compared to control values							
	Reticulocyte ribosomes				Bone marrow ribosomes			
Chloramphenicol (μmoles/ml)	^{14}C-leucine (cpm/mg)	(%)	^{14}C-phenyl-alanine (cpm/mg)	(%)	^{14}C-leucine B.M. 1 (cpm/mg)	(%)	^{14}C-leucine B.M. 2 (cpm/mg)	(%)
None (control)	910	100	4,050	100	3,960	100	4,960	100
0·05	829	93			2,800	71	3,800	77
0·5	756	83	3,120	77	2,530	64	3,100	63
1·0	464	51	1,793	45	1,935	49	2,262	46
2·0	407	45					233	5

* The incubation mixture consisted of 1·0 ml containing 0·2 mg pH 5-enzyme protein, 0·28 mg rabbit reticulocyte ribosomal protein or 0·03 mg rabbit bone marrow ribosomal protein, 20 μg pyruvate kinase, 1 mg glucose, 600 μg potassium penicillin G, and the following, in μmoles: magnesium acetate, 10; potassium chloride, 50; pH 7·8 Tris–HCl, 100; 2-mercaptoethanol, 6; mixture of 18 amino acids, 0·05; GTP, UTP, CTP, 0·025; ATP, 1; phosphoenol pyruvate, 5; either DL-leucine-1-^{14}C (7·84 mc/mmole), 540,000 cpm per flask, or L-phenylalanine-U-^{14}C (140 mc/mmole) 607,500 cpm per flask. Incubation at 37° for 14 hr.

bacterial contamination, it is not possible to guarantee success; thus it is conceivable that the results in Table 3 could represent undetected bacterial incorporation of isotope that was inhibited to a greater degree by the higher concentrations of chloramphenicol. We doubt this because even the lowest concentrations of chloramphenicol

are bacteriostatic for most common organisms, and the level of penicillin used also was sufficient to prevent the growth of most (but not all) bacteria. Another major objection to acceptance of this experiment is the absence of data to indicate that mammalian ribosomes will maintain their incorporating ability for such a long time. This experiment is a preliminary observation and, before it is dismissed as an artifact or accepted as a valid observation, time-course studies should be performed.

DISCUSSION

The discrepancy between the effect of chloramphenicol on bacterial and mammalian cells suggested to von Ehrenstein and Lipmann[7] that at least one step in the protein synthetic pathway is different in the two systems. The clinical reports of the toxicity of chloramphenicol suggested an alternative hypothesis to us, since the predictable (as opposed to idiosyncratic) hematological changes are related both to time and dosage.[20–26] Under optimal conditions, bacteria reproduce in a matter of minutes, whereas mammalian bone marrow cells require many hours for reproduction. Hence, bacterial messenger RNA is necessarily turning over more rapidly than mammalian RNA which apparently binds to the ribosomes more firmly. The usual cell-free incubation system measures the incorporation of a labeled amino acid into a trichloroacetic acid-insoluble material after an incubation period of an hour or less. This brief period resembles the lifetime of the bacterial cell, but it is only a small fraction of the generation time of the mammalian cell and therefore the chances of finding an effect in the former and not in the latter are favored. With longer incubations we found that low levels of mammalian ribosomes are susceptible to chloramphenicol inhibition. Presumably the endogenous m-RNA separates from the ribosome and is prevented from reassociating with it in the presence of chloramphenicol.

A similar situation probably occurs in the case of amino acid incorporation stimulated by m-RNA; the latter turns over relatively slowly in mammalian systems and only a few binding sites for new m-RNA are available. After a preincubation, additional sites become available as m-RNA and ribosomes (or polyribosomes) detach with the completion of the synthesis of a protein molecule, as suggested by Hardesty *et al.*[27] On the next association of a ribosome with a template (messenger) RNA, chloramphenicol may bind either the ribosome or the m-RNA. The studies of Vazquez[28] in *Escherichia coli* indicate that it is the ribosome that binds ^{14}C-chloramphenicol. This conclusion also is supported by the observation of Shaeffer *et al.*[9]. that *E. coli* ribosomes stimulated by rabbit reticulocyte ribosomal RNA still can be inhibited by chloramphenicol. The binding is apparently a loose one, since the chloramphenicol can be washed out.[28]

Large amounts of polyuridylic acid have overcome the block in the stimulated synthesis of polyphenylalanine.[10] Once the available binding sites are filled, additional m-RNA will not stimulate further, and chloramphenicol apparently cannot inhibit. At high levels of RNA, there is frequently some inhibition of incorporation both in our reticulocyte system and in the yeast system of So and Davie.[6] This finding may reflect the occupancy of binding sites by RNA that is not active as a template. Alternatively, it could represent inhibition by DNA or polydeoxyribonucleotides, as suggested by DiGirolamo *et al.*[30] Weisberger *et al.*[31] avoided this possibility by extracting RNA from rabbit reticulocyte ribosomes and using this material to stimulate. Their incorporation was enhanced linearly over the entire range tested, and

inhibition by chloramphenicol was consistently demonstrable. It is not clear to us why the yeast ribosomal system is not inhibited by chloramphenicol, since the lifetime of the cell is as brief as that of many bacteria. Unfortunately, So and Davie did not determine whether the RNA-stimulated synthesis of protein in their yeast ribosome system was susceptible to chloramphenicol.

Addendum: Since this manuscript was submitted, preliminary time course studies have been performed comparing protein synthesis after one hour and sixteen hours of incubation. With low levels of ribosomes pelleted only once (like those in Table 3), ^{14}C-amino acid incorporation was significantly greater at 16 h in the control tubes but failed to increase over the one h level in the chloramphenicol treated incubation mixtures. Twice pelleted ribosomes failed to demonstrate any additional incorporation with prolonged incubation.

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