# CONCERNING THE SITES OF SYNTHESIS OF PROTEINS OF CHLOROPLAST RIBOSOMES AND OF FRACTION I PROTEIN (RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE)

Maurice M. Margulies

Radiation Biology Laboratory, Smithsonian Institution, 12441 Parklawn Drive, Rockville, Maryland 20852

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SUMMARY: Chloramphenicol and spectinomycin selectively inhibit amino acid incorporation into Fraction I protein but have no effect on incorporation into Fraction II protein. Neither antibiotic depresses specific radioactivities of either 70 or 80 S ribosomes. Cycloheximide inhibits incorporation of amino acid into Fraction I and Fraction II proteins equally. The specific radioactivities of 70 S and 80 S ribosomes are depressed equally by cycloheximide. These results suggest that most of the proteins of 70 S chloroplast ribosomes are made outside the chloroplast. They also confirm previous findings that accumulation of ribulose-1,5-diphosphate carboxylase is inhibited by both chloramphenicol and cycloheximide.

## INTRODUCTION

Both chloramphenicol (see 1 for review) and spectinomycin (2,3,4) bind to chloroplast ribosomes and inhibit protein synthesis by chloroplasts. Neither bind to cytoplasm ribosomes or inhibit protein synthesis of the cytoplasm (1,2,3,4). Cycloheximide has no effect on protein synthesis by chloroplasts but inhibits protein synthesis of the cytoplasm (5,6). To further substantiate the specificities of these antibiotics an attempt was made to examine their effects on formation of nascent protein bound to chloroplast 70 S and cytoplasm 80 S ribosomes. These experiments have yielded information concerning the sites of synthesis of the proteins of chloroplast 70 S ribosomes and of ribulose-1,5-diphosphate carboxylase (RuDPC).

## METHODS

A wild type Chlamydomonas reinhardi, mating type plus, was cultured in a mineral medium (7) which contained Tris and $MgSO_4 \cdot 7H_2O$ (0.5 g/l) and was aerated with 5% $CO_2$, 95% air. When the cell concentration reached 2 to 6 x $10^6$ cells/ml, the culture was divided into replicate portions, antibiotic solutions were added, and incubation was continued for 1 hour. Then $^3H$ L-leucine (New England Nuclear) was added (final concentration 0.5mM with a

specific radioactivity of 4 mc/mmole), and incubation was continued for 2 hours (about 0.2 of the time required for cells to double in number). An extract which contained ribosomes was prepared from these cells, essentially as described by Hoober and Blobel (8). Samples (0.2 to 0.8 ml) of the 30,000 x g supernatant fraction from the cell extract were layered onto isokinetic density gradients of sucrose (9), and were centrifuged for 4 hours at 40,000 rpm in a Spinco SW 41 Ti rotor. Each gradient was then fractionated and the absorption (254 nm) along the gradient was measured. Cell suspensions and gradient fractions were analyzed for radioactivity in protein (10), and sometimes for RuDPC activity (11). Alternatively, ribosomes were sedimented from the cell extract by centrifugation and were resuspended and stored at -80° till analyzed by centrifugation. Total radioactivity of 80 S ribosomes is the sum of radioactive protein in the fractions of the 80 S ultraviolet peak, while total radioactivity of 70 S ribosomes is the sum of the radioactive protein in the fractions of the 70 S peak and 60 S shoulder (see footnote below).

## RESULTS AND DISCUSSION

Cycloheximide inhibited amino acid incorporation into total cell protein as follows: 25 percent at 0.1 µg/ml; 50 percent at 0.2 µg/ml; 70 percent at 0.5 µg/ml; 85 to 90 percent at 1 to 20 µg/ml. Cycloheximide (0.5 µg/ml) inhibited amino acid incorporation into Fraction I protein, Fraction II protein, and 70 S ribosomes partially and equally (Tables I and II). Inhibition of amino acid incorporation into 80 S ribosomes appears greater (Table II), because fewer 80 S ribosomes were recovered from cells that had

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In absorbance scans, a large peak was detected about two-thirds of the way from the top of the density gradient. If this peak was given a value of 80 S (8), then a second smaller peak had a value of 70 S. When stored ribosomes were used, the 80 S peak had the same absorbance and location, but there was a shoulder at 55 to 60 S in addition to the 70 S peak. A large absorption peak also occurred in the green portion at the top of the gradient. With stored ribosomes there was a very small absorbance peak at about 20 S which coincided with both a peak of radioactive protein and with the distribution of RuDPC activity. This 20 S peak is referred to as Fraction I and is probably mainly RuDPC (12). A second peak of radioactive protein occurred nearer the top of the gradient and had a value of less than 10 S. It is referred to as Fraction II. These fractions presumably correspond to leaf Fractions I and II (12,13). They were only resolved if stored ribosomes were examined.

Table I. Effect of chloramphenicol, spectinomycin and cycloheximide on amino acid incorporation into Fraction I and Fraction II proteins.

| Additions | Gradient region | | | | Cpm in fraction I / Cpm in fraction II |
|---|---|---|---|---|---|
| | Fraction I | | Fraction II | | |
| | Cpm | Inhibition Percent | Cpm | Inhibition Percent | |
| None | 370 | -- | 501 | -- | 0.74 |
| Chlor(100)+ | 206 | 44 | 588 | -17 | 0.35 |
| None | 309 | -- | 433 | -- | 0.72 |
| Spec(20.)+ | 219 | 29 | 395 | 9 | 0.56 |
| None | 556 | -- | 694 | -- | 0.80 |
| Cyc(0.5)+ | 258 | 53 | 327 | 53 | 0.79 |

+Abbreviations used are: Chlor = chloramphenicol; Spec = spectinomycin; Cyc = cycloheximide. The numbers in parentheses are concentrations in µg/ml.

been treated with cycloheximide. However, if specific radioactivities of 70 and 80 S ribosomes were compared, cycloheximide depressed amino acid incorporation into both equally (Tables II and III).

Chloramphenicol inhibited amino acid incorporation into total cell protein about 20 percent at concentrations of 10 to 200 µg/ml. Chloramphenicol (100 µg/ml), did not inhibit amino acid incorporation into Fraction II protein nor into 80 S ribosomes (Tables I and II). However, the radioactivity recovered in Fraction I protein and in 70 S ribosomes was decreased equally. But, since fewer 70 S ribosomes were recovered from cells that had been incubated with chloramphenicol and labeled amino acid, the specific radioactivity of 70 S ribosomes was not depressed significantly (Tables II and III). Thus, the effects of chloramphenicol on the decreased recovery of 70 S ribosomes may be separated from the effects of chloramphenicol on incorporation of amino acids into proteins of 70 S ribosomes. The decreased recovery

Table II. Effect of chloramphenicol, spectinomycin and cycloheximide on amino acid incorporation into ribosomes.

| Additions[+] | Gradient region[++] | | | | | |
|---|---|---|---|---|---|---|
| | 70 S | | | 80 S | | |
| | Radioactivity (cpm) | Ribosomes (relative amounts) | Specific radioactivity | Radioactivity (cpm) | Ribosomes (relative amounts) | Specific radioactivity |
| None | 708 | 72 | 9.8 | 1,400 | 180 | 7.8 |
| Chlor(100) | 370(48) | 42(42) | 8.8 | 1,380(1) | 193(-4) | 7.2 |
| None | 484 | 45 | 10.8 | 806 | 122 | 6.6 |
| Spec(10) | 256(45) | 28(38) | 9.2 | 745(8) | 113(7) | 6.6 |
| Spec(20) | 318(34) | 24(47) | 13.2 | 796(1) | 98(20) | 8.1 |
| None | 890 | 106 | 8.4 | 902 | 156 | 5.8 |
| Cyc(0.2) | 672(24) | 106(0) | 6.4 | 552(39) | 111(29) | 5.0 |
| Cyc(0.5) | 356(60) | 110(-4) | 3.2 | 241(73) | 95(39) | 2.5 |

[+] See footnote to Table I.
[++] Numbers in parentheses are inhibition in percent.

Table III. Effect of chloramphenicol, spectinomycin and cycloheximide on specific radioactivities of 70 S and 80 S ribosomes.[+]

| Additions | Specific radioactivity, percent inhibition | |
|---|---|---|
| | 70 S ribosomes | 80 S ribosomes |
| Chlor(100) | 10 | 8 |
| Spec(20) | -22 | -23 |
| Cyc(0.5) | 62 | 57 |

[+] Data are derived from Table II.

of monomers of 70 S ribosomes from cells treated with chloramphenicol, and of monomers of 80 S ribosomes from cells treated with cycloheximide may be due to the accumulation of corresponding polysomes (8). The effects of spectino-

mycin on the quantity of recoverable 70 S ribosomes, on the specific radioactivity of 70 S ribosomes (Tables II and III), and on amino acid incorporation into Fractions I and II proteins (Tables I and II) are the same as chloramphenicol.

Our interpretation of these results depends on the finding that most of the radioactivity associated with the ribosomes is in the proteins of the ribosomes and not in nascent protein. The following observations support this conclusion: 1) The fraction of total cellular radioactivity in protein which was associated with crude preparations of ribosomes was approximately constant for labeling periods of 0.5 to 4 hours; 2) Label found in ribosomes was removed very slowly when cells were transferred from growth medium with radioactive amino acid to growth medium with non-radioactive amino acid; 3) The radioactivity associated with ribosomes was not decreased when puromycin was added to labeled cells or to extracts from labeled cells. It is suggested that much (if not all) of the protein of chloroplast ribosomes is synthesized by 80 S cytoplasm ribosomes. Cycloheximide (see Introduction), should not inhibit amino acid incorporation into proteins of 70 S chloroplast ribosomes unless some of these proteins are made on 80 S ribosomes. After correction was made for the decreased number of 80 S ribosomes found in extracts of cells treated with cycloheximide, it was shown to inhibit equally amino acid incorporation into 70 S and 80 S ribosomes (Tables II and III). This result was obtained with 0.5 µg/ml cycloheximide, a concentration lower than that required to inhibit total cell protein synthesis maximally. Chloramphenicol (see Introduction) should not inhibit amino acid incorporation into proteins of 70 S ribosomes unless some of these proteins are made on 70 S ribosomes. After correction was made for the decreased number of 70 S ribosomes found in extracts of cells treated with chloramphenicol, it was found that chloramphenicol did not inhibit amino acid incorporation into protein of either 70 or 80 S ribosomes (Tables II and III). These results were obtained with 100 µg/ml of chloramphenicol, a concentration greater than that required to inhibit total

cell protein synthesis maximally. Thus it appears that cycloheximide but not chloramphenicol inhibit the synthesis of proteins of 70 S ribosomes. It can be concluded from this that most of the proteins of chloroplast ribosomes are made outside the chloroplast on 80 S ribosomes. Since the RNA of chloroplast ribosomes is synthesized in the chloroplast (14) and the protein of chloroplast ribosomes is synthesized in the cytoplasm, the synthesis of chloroplast ribosomes is a task requiring cooperation of chloroplast and cytoplasm. Our data agree with previous reports that synthesis of RuDPC is inhibited by both chloramphenicol and cycloheximide (4,15,16,17). This dual inhibition suggests that both chloroplast and cytoplasm participate in the synthesis of RuDPC. More extensive experiments (17) which show a reciprocal selective inhibition of chloramphenicol and cycloheximide for incorporation of amino acid into each subunit of RuDPC support and extend this view. Such studies point to a complex interrelationship between the chloroplast and the rest of the cell for synthesis of the macromolecular constituents of the chloroplast.

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