DIFFERENTIAL SYNTHESIS OF RIBULOSEDIPHOSPHATE CARBOXYLASE SUBUNITS*

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SUMMARY. Chloramphenicol specifically inhibited the synthesis of the large subunit of ribulosediphosphate carboxylase. Cycloheximide exerted a primary effect upon synthesis of the smaller subunit and influenced production of the larger subunit by rapidly inhibiting total protein synthesis.

The synthesis of ribulosediphosphate carboxylase (RudPCase) is inhibited by chloramphenicol (CAP), an inhibitor of protein synthesis on chloroplast ribosomes (Smillie et al., 1967; Spencer, 1965). The development of RudPCase activity in greening plants (Keller and Huffaker, 1967) is also inhibited by both CAP and cycloheximide (CHI), an inhibitor of protein synthesis on cytoplasmic ribosomes of plants. Such studies have been used in combination to suggest that the chloroplast may be a site of synthesis of RudPCase (Smillie et al., 1967).

Kawashima (1970) recently showed that RudPCase subunits may be labeled unevenly when radioactive CO_2 is pulsed into tobacco plants for following the distribution of label into the major subunits. While a number of interpretations of these data are possible, since factors such as variations in precursor pool size cannot be evaluated, these experiments may also suggest differential modes or sites of synthesis of the two enzyme subunits.

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In the experiments discussed here, inhibitors of protein synthesis were used to test whether differences do occur in the mode of synthesis of RudPCase subunits in barley plants. Incorporation of labeled amino acids into the subunits was followed during the rapid greening phase that follows illumination of etiolated plants.

EXPERIMENTAL PROCEDURE.

Plant materials. Barley plants (Hordeum vulgare L. var. 'Blanco Mariout') were grown for 7 days in the dark at 24° and 55% relative humidity (Travis et al., 1969).

Incorporation of amino acids and antibiotics. Twelve cm (tip to base) of the first leaf of 15 seedlings was excised and placed base down in 10 ml of an aqueous solution of 30 mM $MgCl_2$ and 50 mM NH_4NO_3 (Kleinkopf et al., 1970). The leaves were then placed in the light (21,000 lux) for 3 to 6 hours as indicated in each experiment. Appropriate inhibitors (CAP, 100 µg; and CHI, 1, 2, 4, and 8 µg) and labeled amino acids (^{14}C-leucine, 50 µc, and ^{3}H-leucine, 90 µc) were added simultaneously in one ml of the incubation mixture, and incorporation was allowed to proceed for an additional 3 or 6 hours.

Enzyme purification. RudPCase was isolated and purified as previously described, and incorporation of radioactivity into enzyme protein was determined by analysis of label in a specific antibody precipitate (Kleinkopf et al., 1970). Protein was determined by the method of Lowry et al. (1951).

Separation of enzyme subunits. The major size classes of RudPCase subunits were separated by two procedures. Gel electrophoresis in sodium dodecyl sulfate was used as described by Rutner and Lane (1967) except that the enzyme was first sulfonated with sodium sulfite (Chan, 1968). Alternatively, the subunits were separated on a Sephadex G-100 column at alkaline pH. Before addition to the column the protein was dissociated into subunits by incubation at 37° for 30 minutes in 0.01 M sodium phosphate buffer, pH 11.3, containing 0.1 M NaCl (Wildman and Kawashima, 1970).

Experimental design. In all experiments, two containers, each containing 1.5 gm of leaf tissue, were paired, one receiving ^{14}C-leucine and the other ^{3}H-leucine in the incubation solution. When inhibitors were added, they were always added simultaneously with the ^{14}C-leucine. At the end of the period of illumination, leaves from each ^{14}C- and ^{3}H-leucine sample pair were combined and homogenized, and the enzyme was isolated. Thus, each isolated enzyme preparation contained a mixture of ^{14}C- and ^{3}H-labeled molecules. When inhibitors were added along with ^{14}C-leucine to the incubation mixture, inhibition of protein synthesis would be detected as a decrease in the amount of ^{14}C relative to ^{3}H in the purified enzyme preparation. Also, if an inhibitor exerts a greater effect on incorporation of amino acids into one subunit than into another, this will be indicated by a decrease in the ^{14}C/^{3}H ratio of the sensitive subunit.

RESULTS. Incorporation into RudPCase showed a distinct pattern of inhibition. CAP and CHI cause a marked decrease in the ^{14}C/^{3}H ratio of the enzyme specifically precipitated by antibody (Table I).

TABLE I

Incorporation of Radioactive Leucine Into Antibody Precipiated RudPCase

	^{3}H CPM	^{14}C CPM	^{14}C/^{3}H	µg protein
No inhibitors	1720	1570	0.91	18
Chloramphenicol (100 µg/ml)	1512	950	0.63	21
Cycloheximide (2 µg/ml)	1060	985	0.93	15
(4 µg/ml)	880	678	0.77	16
(8 µg/ml)	910	600	0.66	15

Experimental conditions: Detached barley leaves were placed in light for 6 hours. Labeled leucine and inhibitors were then added to the solutions, and incorporation was allowed to proceed for another 3 hours.

The differential effects of CAP on incorporation into the subunits is graphically illustrated by separation of the enzyme subunits on Sephadex G-100 columns. The decreased $^{14}C/^{3}H$ ratio in the large subunit shows inhibition of synthesis by CAP (Fig. 2) in comparison with the control (Fig. 1). CHI, in contrast, preferentially inhibits formation of the smaller subunit (Fig. 3). Results were similar when the subunits were separated on polyacrylamide gels.

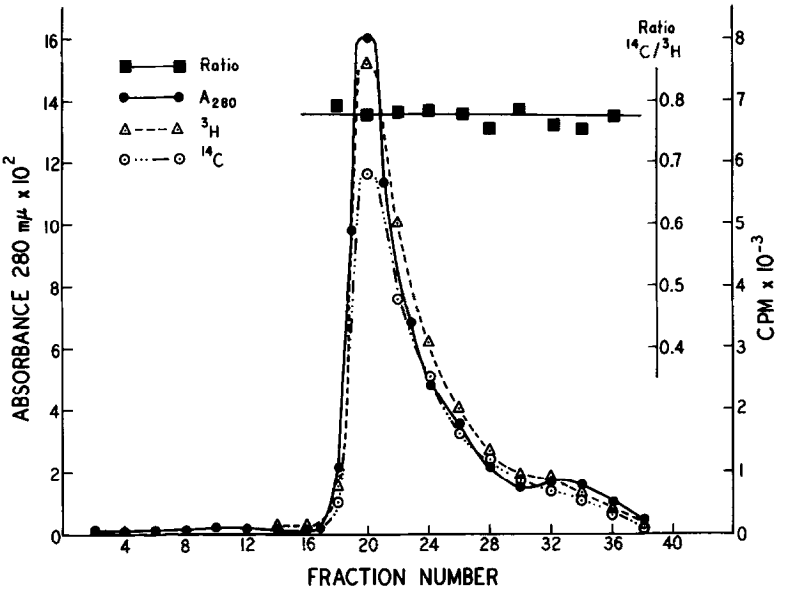

Figure 1. Fractionation of enzyme subunits on Sephadex G-100 from control solution with no antibiotics added during 3-hour incubation with labeled amino acids. Elution from the column was monitored by observing A_{280}, -o-o-o; cpm for ^{3}H, -Δ-Δ-Δ-; and cpm for ^{14}C -o-o-o-. The ratio for ^{14}C relative to that for ^{3}H was calculated as shown -□-□-□-.

At high concentrations of CHI (4 μg/ml and 8 μg/ml), where the inhibition of formation of enzyme is nearly complete, it was not possible to detect a differential level of inhibition in the two chains. Only when inhibition was partial (1-2 μg/ml) was this effect observed. At a CHI concentration of 2 μg/ml, when ^{14}C-leucine incorporation into whole enzyme is inhibited 30% relative to the control, the smaller of the two subunits is inhibited nearly 50%.

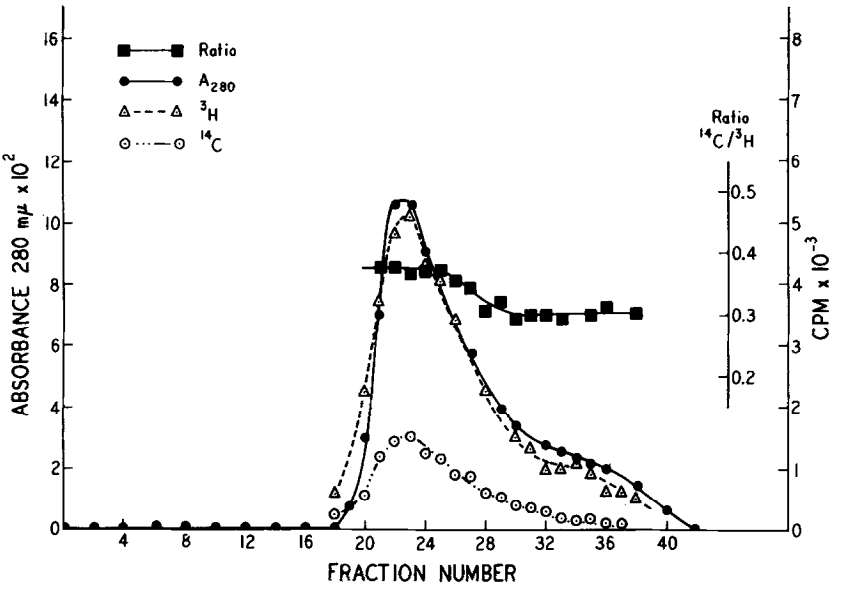

Figure 2. Fractionation of enzyme subunits on Sephadex G-100 from solution with CAP added during 3-hour incubation with labeled amino acids. Curves are as indicated in Fig. 1.

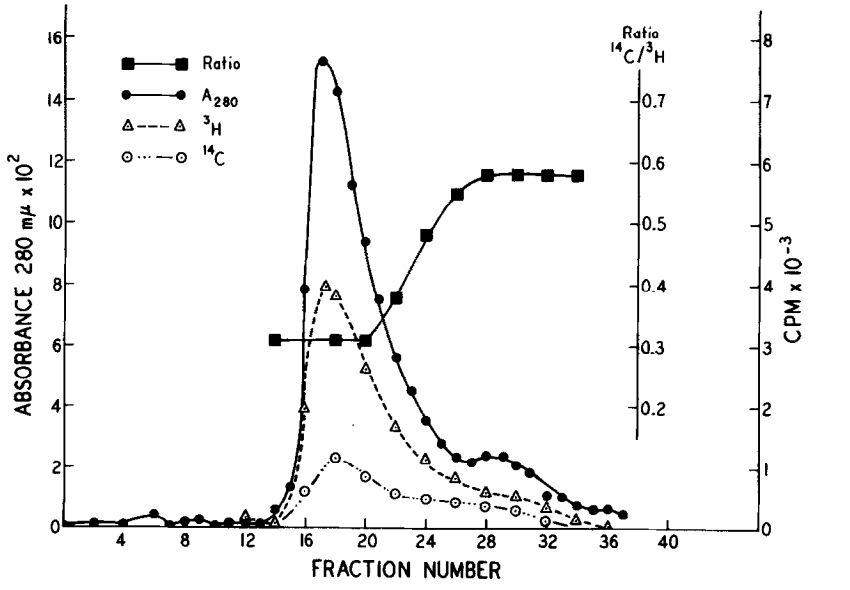

Figure 3. Fractionation of RudPCase subunits on Sephadex G-100 from solution with cycloheximide (2 μg/ml) added during 3-hour incubation of plants with labeled amino acids. Curves are as indicated in Fig. 1.

DISCUSSION. These studies support the conclusion that the two subunits of RudPCase are synthesized at separate locations in the cell. Since CAP markedly inhibits synthesis of the larger of the two subunits, this subunit may be made within the chloroplasts. Since synthesis of the smaller subunit is much less sensitive to CAP, its production is analogous to that of the majority of soluble enzymes in the leaf tissue. Hence, the site of synthesis of this protein may be in the cytoplasm with subsequent transport into the chloroplast, where it is united with the larger chains. The results with cycloheximide support this hypothesis. Alternatively, one could account for these observations by postulating the presence of more than one class of chloroplast ribosomes with differing levels of CAP sensitivity, but no available information suggests that such is the case.

The studies with CHI indicate preferential inhibition of formation of the small chain of RudPCase and thus suggest that this subunit is made on a separate class of "cytoplasmic-type" ribosomes. The strong inhibition of amino acid incorporation into both chains of RudPCase by high concentrations of CHI suggests, however, that this inhibitor also exerts a secondary effect by stopping synthesis of the large subunit, possibly by inhibition of the production of some factor enabling chloroplast protein synthesis to proceed.

It is not possible to extrapolate from these observations to a conclusion as to the site of production of messenger RNA which directs the polypeptide synthesis. Even assuming that the respective loci of synthesis for the two chains are the chloroplasts and the microsomal fraction, it is still possible for relatively stable messengers from either a nuclear or chlorplast source to be involved in directing this synthesis. The answer to this question will come only from more refined chemical and genetic studies.

REFERENCES

1. Chan, W. W. C., Biochem. 7, 4247 (1968).
2. Kawashima, N., Biochem. Biophys. Res. Comm. 38, 119 (1970).
3. Keller, C. J. and Huffaker, R. C., Plant Physiol. 42, 1277 (1967).
4. Kleinkopf, G. E., Huffaker, R. C., and Matheson, A., Plant Physiol. in press (1970).
5. Lowry, O. H., Rosebrough, J. J., Farr, A. C., and Randall, J., J. Biol. Chem. 193, 265 (1951).
6. Rutner, A. C., and Lane, D. M., Biochem. Biophys. Res. Comm. 28, 531, (1967).
7. Smillie, R. M., Graham, D., Dioyer, M. R., Grieve, A., and Tobin, N. F., Biochem. Biophys. Res. Comm. 28, 604 (1967).
8. Spencer, O., Arch. Biochem. Biophys. 111, 381 (1965).
9. Travis, R. L., Jordan, W. R., and Huffaker, R. C., Plant Physiol. 44, 1150 (1969).
10. Wildman, S. and Kawashima, N., Personal Communication (1970).