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Cycloheximide Inhibition of Amino Acid Uptake and Protein Biosynthesis by Chloroplasts Incubated *in vitro*

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J. A. LOZANO and D. E. GRIFFITHS. *Cycloheximide Inhibition of Amino Acid Uptake and Protein Biosynthesis by Chloroplasts Incubated* in vitro. R. esp. Fisiol., 27, 29-34.

Cycloheximide is known to be a strong inhibitor of protein biosynthesis in systems with 80 S ribosomes whereas systems with 70 S ribosomes such as isolated mitochondria of isolated chloroplasts are unaffected by this antibiotic. Surprisingly, using a system of isolated chloroplasts from young pea leaves, cycloheximide and N-ethylmaleimide inhibited protein synthesis as measured by incorporation of (^{14}C)-leucine into protein. Experiments performed in several conditions showed that the inhibitory effects of cycloheximide, N-ethylmaleimide and 2,4-dinitrophenol could be produced by an interference with and ATP-dependent transport of one or more amino acids into chloroplasts.

In contrast to chloramphenicol, cycloheximide is known to be strong inhibitor of protein biosynthesis in systems with 80 S ribosomes (24) whereas systems with 70 S ribosomes such as bacteria (7), isolated mitochondria (1, 3, 4, 6, 13, 22, 23) and isolated chloroplasts (20, 25) are unaffected by this antibiotic. In consequence, cycloheximide has been used to differentiate between the relative rates of protein synthesis *in vivo* catalysed by microsomes and chloroplasts or mitochondria (25, 27). With isolated mitochondria conflicting results have been obtained, some authors claiming inhibition of mitochondrial protein synthesis (11) and others a stimulation of amino acid incorporación in the presence of cycloheximide (14).

Some recent reports (1, 2, 11, 12, 16, 21, 23) indicate that cycloheximide is an effective inhibitor of *in vivo* amino acid incorporation into insoluble mitochondrial protein, which is supposed to be synthesized within the mitochondria. Several possible interpretations are given in these papers.

According to Smillie *et al.* (25) chloroplast ribosomes are known to be the sites of protein synthesis of the photosynthetic electron transfer pathway and their *in vivo* synthesis is inhibited by chloramphenicol

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and, surprisingly, by cycloheximide. SMILLIE *et al.* suggested that the inhibitory effect of cycloheximide is indirect and resulted from a block in the synthesis of certain structural elements essential for the synthesis of the electron transfer proteins and their subsequent incorporation into lamellae.

Recently, MARGULIES and PARENTI (18) showed that ribulose-1,5-diphosphate carboxylase is synthesized by isolated bean chloroplasts. On the other hand, FILNER and KLEIN (8) *in vivo* experiments with bean plants claimed that cycloheximide blocked the increase of chloroplast ribulose carboxylase activity that normally occurs when etiolated bean seedlings are briefly illuminated.

Using a system of isolated chloroplasts from young pea leaves we have found that cycloheximide inhibited protein synthesis as measured by incorporation of (^{14}C)-leucine into protein. Contamination by nuclei, whole cells and cytoribosomes could not explain the inhibition obtained when cycloheximide was used. In view of the conflicting reports the inhibition of amino acid incorporation into chloroplasts by cycloheximide has been further investigated.

Materials and Methods

Methods for the preparation of chloroplasts, incorporation of amino acids and scintillation counting have been described in detail previously (9, 10, 15).

Chloroplasts were prepared from pea leaves (4 days old) with a slight modification of the method described by PARENTI and MARGULIES (19). Chloroplasts were washed once with Honda medium (26). The assay of incorporation was based on that of SPENCER and WILDMAN (26) but the final reaction mixture was 0,3 ml and consisted of 0.2 ml of chloroplast preparation together with: 12 mM $MgCl_2$, 100 mM KCl, 16 mM mercaptoethanol, 2.75 mM ATP*, 8.3 mM phosphoenolpyruvate, 10 μg of pyruvate kinase, 0.13 mM UTP, 0.13 mM GTP, 0.13 mM CTP, a mixture of 2 μg of each of twenty amino acids excepting leucine. The incorporation reaction was started by adding (^{14}C)-leucine (10 μl) of a solution containing 10 μC and 0.066 μmole per ml. Temperature was 25° C. The preparation of the samples for counting was carried out as described by MANS and NOVELLI (17) placing 0.1 ml of incubation medium after the periods of incubation in each disc of paper. Counting was effected with a Packard Tri-Carb liquid scintillation spectrometer with 56 % efficiency.

Control experiments showed that contamination of the chloroplast preparation by nuclei, whole cells and cytoribosomes was responsible for less than 5 % of the total (^{14}C)-leucine incorporation into protein (15). This degree of contamination could not explain the inhibitory effect of cycloheximide.

Results and Discussion

Table I shows that the inhibition produced by cycloheximide increases with the time. In addition, protein synthesis by isolated chloroplasts is highly dependent on

Table I. *Time course of the inhibition produced by cycloheximide.*
Incubation time was 60 minutes. Temperature was 25° C.

System	RADIOACTIVITY: counts/min. sample		
	Time: minutes		
	0-30	30-45	45-60
Normal	880	210	75
Normal + CH (8 μg/ml)	620	110	30
Inhibition %	29	47	60

* *Abbreviations:* ATP, adenosine triphosphate; CH, cycloheximide; CTP, cytidine triphosphate; DNP, 2,4-dinitrophenol; NEM, N-ethylmaleimide; GTP, guanosine triphosphate; UTP, uridine triphosphate.

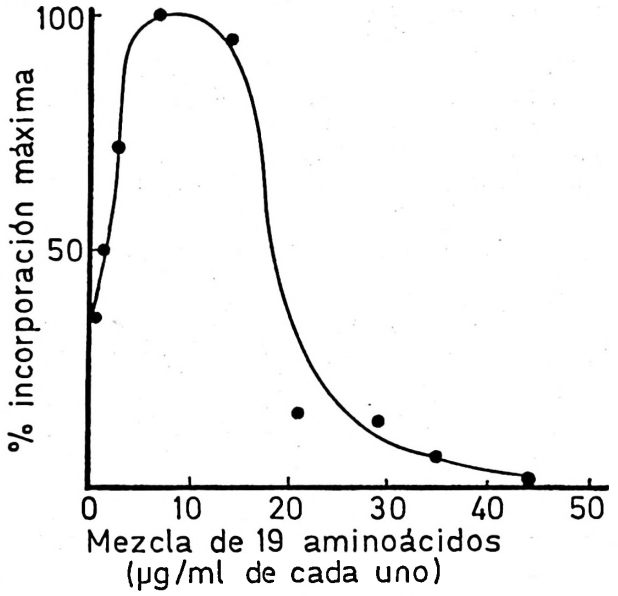

Fig. 1. *Incorporation of (^{14}C)-leucine in presence of unlabelled amino acids.*
(This figure is reproduced from LOZANO and GRIFFITHS [15]).

the addition of a mixture of amino acids (15) (Fig. 1).

In order to verify that the inhibitory effect of cycloheximide could be produced by an interference with a hypothetic transport of one or more amino acids into chloroplasts, the chloroplasts were incubated, in the presence of different concentrations of amino acids, with cycloheximide or N-ethyl maleimide, a known inhibitor of transport processes.

Data shown in Figure 2 suggest the possible existence of a such transport process. The optimum inhibition was obtained when the amino acid mixture was present in the medium at standard concentration (6.66 µg/ml of each of nineteen amino acids). The inhibition or activation produced by cycloheximide or N-ethylmaleimide was strongly dependent on the concentration of the amino acid mixture present in the medium. So, cycloheximide and N-ethyl maleimide did not interfere the amino acid incorporating activity if the amino acid mixture was absent in the medium. These data indicate that these substances did not affect the protein synthesis, suggesting that their effect was dependent on the presence of the amino acid mixture necessary for an optimun protein synthesis. Relatively high amino acid concentrations diminished the incorporation of (^{14}C)-leucine into protein (Fig. 1), but the addition of cycloheximide returned the system to a high level of incorporation (Fig. 2). This fact would be in agreement with the interpretation that cycloheximide (or N-ethyl maleimide) decreases the actual concentration of one or more amino acids within the chloroplasts due to a block of transport of amino acids. It is tempting to suggest that cycloheximide and N-ethylmaleimide affect only the process of an ATP-dependent transport of amino acids into chloroplasts.

When cycloheximide was added after preincubation with ATP and the amino acid mixture, as shown in table II, no inhibitory effect was present.

2,4-dinitrophenol is a known substance affecting the energy metabolism. In table III is shown the inhibitory effect of DNP on the protein synthesis carried out

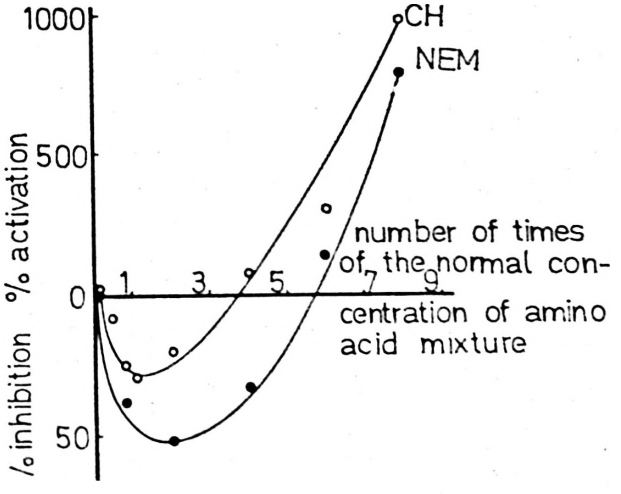

Fig. 2. *Effect of CH (8 µg/ml) and NEM (62 µg/ml) on (^{14}C)-leucine incorporation at various cold amino acid mixture concentrations.* Normal concentration of cold amino acid mixture corresponds to the maximum of figure 1: 6.6 µg/ml of each one of the nineteen amino acids.

Table II. *Effect of different preincubations on the inhibitory action of cycloheximide.* Preincubation standard medium (PSM) was similar to incubation medium described under MATERIAL AND METHODS, but without addition of (^{14}C)-leucine ATP and cold amino acid mixture. Preincubations were performed at 25° C for 10 min. Incubation time was 60 min started with the addition of (^{14}C)-leucine. Others substances, such as ATP and/or CH and/or cold amino acid mixture, were added at the beginning of incubation time.

Addition to the PSM	RADIOACTIVE PROTEINS: counts/min		Inhibit. %
	cycloheximide		
	added	not added	
No preincubation	660	480	27
Preincubation:			
ATP + amino acid mixture	235	355	—50
ATP (Amino acid mixture added after preincubation)	225	167	26
Amino acid mixture (ATP added after preincubation)	440	260	42
ATP (No amino acid mixture added during preincubation or incubation time)	136	144	—6

Table III. *Effect of DNP (10^{-4} M) on amino acid incorporation.* Preincubation standard medium (PSM) and other details are described in table II. Ultrasonic broken chloroplasts were prepared as described by GRIFFITHS and LOZANO (9).

Condition		Radioactive proteins counts/min	Inhibit. %
Addition to PSM	Addition to the incubation medium		
Whole chloroplasts			
—	ATP	308	—
DNP	ATP	264	14
—	ATP+AAM *	669	—
DNP	ATP+AAM	419	37
ATP	—	150	—
ATP	DNP	155	0
ATP	AAM	610	—
ATP	DNP+AAM	573	6
Broken chloroplasts			
—	ATP	153	—
DNP	ATP	151	1
—	ATP+AAM	214	—
DNP	ATP+AAM	224	0 **
ATP	—	71	—
ATP	DNP	73	0 **
ATP	AAM	194	—
ATP	DNP+AAM	191	2

* AAM = (^{12}C)-amino acid mixture.
** Very slight activation.

by isolated chloroplasts. As can be seen, inhibition produced by addition of DNP in different conditions, was absent in ultrasonic broken chloroplasts or in whole chloroplasts without addition of cold amino acid mixture. These data suggest that DNP inhibition affects an ATP-dependent transport of amino acids into chloroplasts.

We can conclude that there is evidence for active transport of amino acids into isolated chloroplasts from young pea leaves. This transport is inhibited by cycloheximide. Experiments are presently under way to obtain knowledge about the nature of this transport.

Recently, BUCHANAN *et al.* (5) showed the existence of a leucine transport by rat liver mitochondria *in vitro*, but they did not study the effect of cycloheximide on the transport. The use of cycloheximide as a specific inhibitor of cytoribosomal protein synthesis should be treated with caution.

Resumen

La cicloheximida se considera un fuerte inhibidor de la biosíntesis de proteínas en sistemas con ribosomas 80 S, mientras que los sistemas de ribosomas 70 S tales como mitocondrias o cloroplastos aislados no se afectan por este antibiótico. En un sistema de cloroplastos aislados de hojas jóvenes de guisantes se encontró que tanto cicloheximida como N-etil maleimida inhibieron la síntesis de proteínas, medida como incorporación de leucina-C^{14} en

forma de proteínas radioactivas. Distintos tipos de ensayos muestran que los efectos inhibidores de esas sustancias y del 2,4-dinitrofenol pueden deberse a su acción inhibidora sobre un sistema de transporte de aminoácidos al interior de cloroplastos que depende de ATP.

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