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In vivo and *in vitro* Protein Synthesis by Pea Chloroplasts

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The study of the various factors which affect the chloroplast protein synthesis by making use of a pea chloroplast system *in vitro* led to values of synthesis which represent al least 14 % of the value of the synthesis of the *in vivo* system, according to the data deduced from chloramphenicol inhibition.

From the evidence presented during the last few years it is obvious that the chloroplasts are not autonomous bodies with capacity for self reproduction. It is likely, however, that they have some degree of autonomy. Synthesis of the chloroplast structural protein and other membrane-bound and soluble proteins are, partially at least, under the control of chloroplast DNA and these proteins are synthesized inside the chloroplasts themselves. Specific 70 S ribosomes participate in the protein synthesis by chloroplasts.

The primary purpose of research on cellular processes is not to obtain high rates in the processes, but to elucidate their biochemical and biophysical mechanisms. In the early stages of the problem it is normal to find low yields on the *in vitro* assays. For instance, the velocity of assimilation of carbon dioxide by isolated chloroplasts was multiplied by a factor of 30 from the early results obtained by ALLEN *et al.* (1) to the last ones (16).

We have already published several papers in connection with *in vitro* synthesis of proteins by chloroplasts isolated from young pea leaves. The control of factors related with the incubation medium and with the structure of the chloroplasts gave high incorporations of amino acids as proteins. In most of the cases our results were quantitatively more significant than others obtained with similar systems. Accordingly, we tried to evaluate the relationship between the synthesis of chloroplast proteins of pea leaves *in vivo* and *in vitro*.

PARENTI and MARGULIES (22) working with chloroplasts isolated fom strongly illuminated bean leaves have shown a ratio of 1 to 1,000 between *in vitro* to *in*

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vivo values. Previously leaves were in etiolated condition. In the literature there is not very much information about the problem in hand.

Materials and Methods

Growing and harvesting of plants, chloroplast isolement, [^{14}C]-leucine incorporation into proteins, radioactivity measurement, estimation of contaminations, chlorophyll and protein determinations and other experimental aspects were performed as described previously (13, 14, 17, 18).

For the analysis of total chlorophyll in pea-leaves, an extraction with 80 % acetone was made after grinding the material with a teflon pestle homogenizer chilled with ice during 10 minutes.

When necessary, a 4 mg chloramphenicol/ml solution was supplied to the 4 days-old plants in several (4-5) sprays for 24 hours.

Results and Discussion

In addition to other factors previously studied, we have considered the effect of leucine concentration in the incubation medium on the protein synthesis. Different amounts of a 1.33 mM solution of [^{12}C]-leucine were added to the incubation media. Results are shown in Figure 1. The [^{12}C]-leucine contained into the chloroplasts, before the incubation process, was judged as negligible.

The leucine incorporation obtained can be related to the total proteins of chloroplasts, because according to our results, in the chloroplasts isolated from pea-leaves of 4-5 days-old there is a protein/chlorophyll ratio of 10/1. This is in agreement with experiments performed on other materials such as wheat (2) and bean (5). In summary, we can establish an *in vitro* amino acid incorporation of 240 pM of leucine/mg chloroplast protein per hour.

In order to estimate the real velocity of

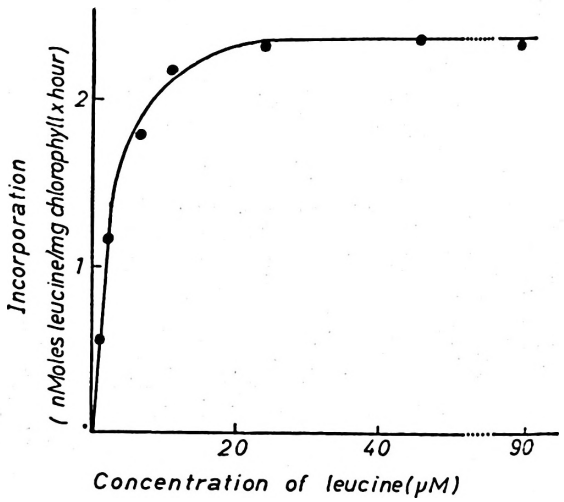

Fig. 1. *Effect of leucine on amino acid incorporation* in vitro.

in vitro synthesis, it is necessary to consider that during the incubation of chloroplasts, they lose by structural damages their ability to synthesize proteins (20). This process is also affected by the presence of ATP (13). In these circunstances, it is advisable to follow only the first 15 minutes of synthesis which yield an incorporation of 374 pM of leucine/mg chloroplast protein per hour.

We can evaluate that leucine represents 10 % of the weight of proteins synthesized by chloroplasts, in agreement with the knowledge about composition of structural protein (11) which is synthesized inside the chloroplasts. That percentage also corresponds to the figures deduced from chloroplast protein composition and from relative incorporation of amino acids into chloroplast protein (12). Therefore, we can deduce a protein synthesis *in vitro* of 500 ng protein/mg chloroplast protein per hour.

It would be interesting to compare these data with the more significant values from the literature. In table I the figures from references 6, 23, 24, 25 and 28 are doubtful because they correspond to experiments with one or more characteristics which are known actually as result of the

Table. I. *Amino acid incorporation into protein by isolated chloroplasts.*

Material	^{14}C-amino acid	Velocity of incorporation	Protein synthesis as ng P/mg chloroplast P×hour	Reference
Pea	Glycine	39 c.p.m./mg P×2 h	7	24, 25
Pea	Hydrolized P	3600 c.p.m./mg P×1 h	61	23
Acetabularia	Hydrolized P	500000 c.p.m./mg C×1 h	188	12
Acetabularia	Valine	164775 c.p.m./assay×30 min	38	11
Spinach	Valine	3000c.p.m./mg C×35 min	5	26
Spinach	Hydrolized P	7500 c.p.m./mg P×90 min	—	2
E. gracilis	Leucine	430 pM/mg RNA×45 min	782	6
E. gracilis	Mixture	13184 c.p.m./mg P×1 h	—	9
Bean	Leucine	50 pM/mg P×1 h	65	22
Bean	Leucine	200 pM/mg P×1 h	262	5
Tobacco	Leucine	39 c.p.m./mg P×1 h	8174	28
Tobacco	Valine	20 pM/mg P×45 min	30	27
Tobacco	Leucine	1329 c.p.m./assay×30 min	—	8
Tomato	Phenylalanine	32 pM/mg P×45 min	42	15
Wheat	Leucine	139 pM/mgC×10 min	109	3

c.p.m.: counts per minute. C: chlorophyll. P: protein. RNA: ribonucleic acid. h: hour.

presence of bacteria or other contaminations (2, 10, 17). Some of these conditions are: neither dependence on ATP nor other co-factors addition, preparation of chloroplasts in an isotonic sucrose medium, lack of inhibition by adding chloramphenicol, etc.

In other cases of table I (8, 9, 11, 15, 26) analytical values were insufficient to calculate the mg of synthesized protein/g of chloroplast protein per hour. Other times, when dates were not shown (3, 11, 26) we suppose an efficiency of 60 % in the radiactive counter. When amino acids different of leucine were used we made the same calculations as with leucine after taking into account the corrections of molecular weights.

According to the above discussion, the value of 500 ng of protein/mg chloroplast protein per hour is greater than most of the values obtained from other systems without bacterial or other contaminations. So, we think it is possible to compare the *in vitro* and *in vivo* systems.

To evaluate the chloroplast protein synthesis by pea leaves, harvestings of 100 leaves were carried out at the end of the 4th and 5th day, with an interval of 24 hours. Chlorophyll content in the leaves was analyzed. From both kind of leaves isolated chloroplasts were prepared and chlorophyll and protein were determined in them.

In this way, as shown in table II, it was possible to know — before and after a 24 hour period — the total chloroplast protein which is contained in a certain number of plants. Supposing a linear synthesis

Table II. *Synthesis of chloroplast proteins during a 24 hour period.*

	Days	
	4th	5th
Weight of leaves from 100 plants (g)	4.3	7.8
Chlorophyll in leaves (mg/g)	0.92	1.08
Chlorophyll in isolated chloroplasts (mg/ml)	0.037	0.068
Protein in isolated chloroplasts (mg/ml)	0.500	0.580
Total chloroplast proteins (mg)	53.4	71.8

Table III. *Chloramphenicol (CAP) effect on protein and chlorophyll synthesis* in vivo.

	4th day	5th day		Inhibition %
		Without CAP	With CAP	
Weight of leaves from 100 plants (g)	4.7	8.6	8.8	—
Chlorophyll *a* in isolated chloroplasts (mg/ml)	0.028	0.061	0.044	52
Chlorophyll *b* in isolated chloroplasts (mg/ml)	0.012	0.018	0.017	17
Total chlorophyll in isolated chloroplasts (mg/ml)	0.040	0.079	0.061	46
Chloroplast proteins (mg)	58.0	80.0	73.4	30

of proteins during the 24 hour period as noted in leaves (7) the first order kinetic gave a synthesis of 12,000±3,000 ng of protein/mg chloroplast protein per hour, in agreement with results obtained by MARGULIES (19) working with etiolated bean leaves illuminated during a 48 hour period.

From this, we could deduce that *in vivo* synthesis is 24 times superior to *in vitro*. However, many chloroplast proteins are not synthesized inside the chloroplasts by means of the specific 70 S ribosome system. A quantitative approach can be made bearing in mind that chloramphenicol inhibits almost completely, even at very low concentration, the protein synthesis carried out by 70 S ribosome systems, as in chloroplasts. On the other hand chloramphenicol does not prevent protein synthesis being carried out by cytoplasmic 80 S ribosome systems.

When plants were submitted to sprays with a solution of 4 mg chloramphenicol/ml the growing of the plants, as shown in Table III, was not affected, but the *in vivo* chloroplast protein synthesis was inhibited by about 30 %. Similar percentages of inhibition have been reported in bean chloroplasts (19, 21). Obviously this inhibition must be produced by the participation of a 70 S ribosome system in the *in vivo* chloroplast protein synthesis. So, the deduced 500 ng protein/mg of chloroplast protein per hour for the *in vitro* synthesis is 14 % of the 3,600 ng of protein/mg of chloroplast protein per hour of the *in vivo* synthesis.

Another effect related to chloramphenicol addition was the partial prevention of chlorophyll synthesis (about 50 %) which was the consequence of the preferential inhibition of chlorophyll a production, in a similar situation as described by NIKOLAEVA (21).

That value of 14 % previously deduced can be considered as minimum because there are at least two other factors to be included in the problem.

Firstly, we have supposed that isolated chloroplasts do not contain leucine inside them. However, from reports of BOARDMAN *et al.* (4) on tobacco chloroplasts and of BAMJI and JAGENDORF (3) on wheat chloroplasts a content of 8-10 pM leucine/mg chloroplast protein can be taken into account.

On the other hand, in the preparation of isolated pea chloroplasts we have found (13) that they are not always functional, because some of them are broken and they are not active for protein synthesis. Even with chlorosplasts kept at 0° C, protein synthesis decreases rapidly. So, in experiments with isolated chloroplasts the amino acid incorporation figures must be lower that the theoretical activity of them, in terms of their chlorophyll content.

Anyway, the considered pea chloroplast system *in vitro* can be very useful for studying the characteristics of the incorporation of amino acids into chloroplast proteins.

Resumen

El estudio de los diversos factores que afectan la síntesis de proteínas cloroplásticas por un sistema *in vitro* de cloroplastos de guisantes condujo a valores de síntesis que representan al menos el 14 % del valor correspondiente al sistema *in vivo*, como se ha deducido a partir de los datos de inhibición por cloramfenicol.

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