3. CHAMBERLIN, M. AND P. BERG. 1962. DNA-directed synthesis of RNA by an enzyme from *Escherichia coli*. Proc. Natl. Acad. Sci. 48: 81–94.
4. DENNY, F. E. 1926. Hastening the sprouting of dormant potato tubers. Am. J. Botany 13: 118–25.
5. GUTHRIE, J. D. 1939. Role of glutathione in the breaking of the rest period of buds by ethylene chlorohydrin. Contrib. Boyce Thompson Inst. 11: 261–70.
6. HUANG, R. C. AND J. BONNER. 1962. Histone, a suppressor of chromosomal RNA synthesis. Proc. Natl. Acad. Sci. 48: 1216–22.
7. KAWAMATA, J. AND M. IMANISHI. 1961. Mechanism of action of actinomycin D, with special reference to its interaction with DNA. Biken's J. 4: 13–24.
8. MICHENER, D. 1942. Dormancy and apical dominance in potato tubers. Am. J. Botany 29: 558–68.
9. REICH, E. AND E. L. TATUM. 1962. Action of actinomycin D on animal cells and viruses. Proc. Natl. Acad. Sci. 48: 1238–45.
10. ROSA, J. T. 1928. Relation of tuber maturity and of storage factors to potato dormancy. Hilgardia 3: 99–124.
11. TS'O, P. O. P. AND C. SATO. 1959. Synthesis of RNA by plants. Exptl. Cell Res. 17: 227–36.

C^{14} Amino Acid Incorporation by Spinach Chloroplast Preparations [1, 2, 3]

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The chloroplast fraction from higher plants has been reported to incorporate amino acids in vitro (1, 2, 4, 7, 8, 9). We have investigated incorporation by spinach (*Spinacia oleracea*) chloroplast fractions and as yet have been unable to demonstrate clearly that the chloroplasts themselves are responsible for the observed incorporation. A severe and major problem appears to be the prevalence of contamination by bacteria.

A typical time course for incorporation of C^{14}-L-leucine, C^{14}-L-phenylalanine or a mixture of C^{14}-L-amino acids by a chloroplast preparation usually continues for at least 8 hours. Frequently the rate increases after the first 3 or 4 hours. Addition of the other 19 amino acids and amides does not enhance but actually inhibits incorporation of any single amino acid. Evidence that the incorporated amino acid is probably in a peptide linkage includes transfer of the originally labeled amino acid from a trichloracetic acid insoluble to soluble form by either acid hydrolysis or papain or trypsin digestion of the isolated labeled protein. Incorporation is inhibited 75 % or more by 10^{-3} M of either chloramphenicol, streptomycin, puromycin, or arsenate. It is not inhibited by Zephiran chloride (1: 5000), penicillin (500 units/ml), ribonuclease (1 mg/ml), deoxyribonuclease (1 mg/ml), or fluoride (10^{-3} M). Incorporation over this long time period is not dependent upon or increased by adding ATP, an ATP generating system, or various substrates such as glucose, malate, ascorbate, or glycolate.

The reaction appears to require O_2 since incubation under N_2 gives a very large inhibition (table I). The enhancement by light (1, 9) can only be consistently reproduced under conditions of low O_2 tension. Photophosphorylation does not seem to be the basis of the light effect because uncouplers (propylamine) had no effect on the light stimulation. On the other hand substrate amounts of TPN do enhance the effectiveness of light (table I) and in other experiments this effect was not shared by TPNH or by catalytic amounts of TPN. The inhibitor of O_2 evolution, *p*-chlorophenyl-1, 1-dimethylurea (CMU), eliminates the light effect, as does the addition of an O_2 trapping system (glucose and glucose oxidase). It seems certain that stimulation by light under these conditions is likely to be simply another manifestation of the O_2 requirement. The pH optimum for incorporation is very broad, running from pH 4.5 to 8.0. Finally, in agreement with previous work (9) a concentration greater than 40 μmoles leucine per milliliter is necessary to achieve the maximum rate of leucine incorporation.

Since many of the unusual characteristics of the chloroplast fraction incorporation system could be explained if microbial contamination were a serious problem, chloroplast preparations were plated out on

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Table I. *Effect of Light on Incorporation by Chloroplast Fraction under Aerobic and Anaerobic Conditions*

In experiment I, each flask contained 2.5 μg of each of the following L-amino acids (Nutritional Biochemical Corp.), glutamic, lysine, valine, alanine, arginine, phenylalanine, aspartic, isoleucine, proline, threonine, tyrosine, serine, tryptophan, histidine, methionine, cysteine, asparagine, leucine, glutamine, and glycine, as well as 0.5 M sucrose, 0.02 M Tris pH 7.6, 0.01 M $MgCl_2$, 1.6 mg chlorophyll, and 325,000 cpm C^{14} algal protein hydrolysate (New England Nuclear) in a total volume of 2.0 ml. The reaction was run for 60 minutes at 20°. Incorporation was determined by the method of Mans and Novelli (6). In experiment II, each flask contained 2.5 μg of each of the 20 amino acids listed above, and 0.02 M Tris pH 7.6, 0.01 M $MgCl_2$, 162,000 cpm C^{14} algal protein hydrolysate, 750 μg chlorophyll in a total volume of 1 ml. The reaction was run for 75 minutes at 20°. The glucose oxidase system included 100 μg glucose oxidase (Sigma) and 6×10^{-2} M glucose.

Treatment		Gas phase 100% O_2	Air	N_2
		(cpm/mg chlorophyll)		
Expt. I				
...	Dark	5700	6700	520
...	Light	6150	5950	1220
4×10^{-5} M CMU	"	...	6750	680
10^{-3} M Propylamine	"	...	...	1160
Expt. II				
...	Light	...	...	900
4×10^{-5} M CMU + 2×10^{-3} M TPN	"	...	...	720
2×10^{-3} M TPN	"	...	...	2220
2×10^{-3} M TPN + glucose oxidase	"	...	...	1090

Table II. *Relation between Bacterial Contamination and Incorporation by Spinach Chloroplast Fraction*

In experiment I, each flask contained 320 μg chlorophyll, 0.02 M Tris pH 7.6, 0.01 M $MgCl_2$, 33 μg L-leucine, 40,000 cpm C^{14} L-leucine (Volk Chemical Co.) in a total volume of 1.0 ml. The reaction was run under 100% O_2 at 20°. In experiment II, each flask contained the same components as in experiment I except 66 μg L-leucine rather than 33 μg leucine was employed. All samples were plated on nutrient agar after the incorporation incubation was completed. Colony counts were made after 72 hours at 25°. Incorporation was measured by the method of Mans and Novelli (6). In all experiments, both cultured bacteria and chloroplasts were prepared from the same batch of spinach.

	Incorporation (mμ moles Leucine) 0 hr	3 hr	Plate colony counts	Approx amount bacteria added
Expt. I				
1. Control	1.0	16.4	5.5×10^7	...
2. Chloroplasts heated 10 min, 55°	0.67	0.7	1.7×10^4	...
3. " "	1.2	29.8	5.3×10^8	3×10^8
4. " "	3.3	49.0	5×10^9	3×10^9
5. " "	0.58	1.25	...	3×10^9 (heated 10 min, 55°
6. Leaves treated with hypochlorite	0.83	1.35	3.1×10^5	...
7. " "	0.84	22.5	5×10^8	3×10^8
8. " "	1.44	43.0	9.3×10^9	3×10^9
Expt. II				
1. Chloroplasts heated 10 min, 55°	0.83	1.25	8×10^5	...
2. " "	0.92	9.3	4.4×10^8	3×10^8
3. " " (+ 1: 10,000 Zephiran chloride + 500 units penicillin)	0.85	7.8	4.7×10^8	3×10^8
4. " " (run under N_2)	0.80	2.1	...	3×10^8

nutrient agar and colony counts obtained. Bacteria were also plated out on agar containing heat denatured chloroplasts but no increase in the number of colonies resulted so Difco nutrient agar was used routinely. Plates were incubated at 25°. In addition, suspensions of the microbes, obtained by suspending the surface bacteria from the nutrient agar plates, were added back to the system. Table II shows that both the chloroplast preparation and the microbial suspension are inactivated by heating at 55° for 10 minutes. Pretreating the leaves by immersing them in a 2% filtered solution of calcium hypochlorite for 5 minutes and then washing thoroughly in sterile water inactivates the incorporating system. Adding back microbial contamination to either chloroplasts prepared from hypochlorite treated leaves or to heat treated chloroplasts resulted in good rates of incorporation. However, there is not a linear relationship between the amount of contamination as measured by colony counts on nutrient agar and incorporation of C^{14} leucine. Thus, adding 3×10^7 cells to heated chloroplasts did not result in a 10-fold increase in incorporation of C^{14}-leucine over adding 3×10^8 cells, and adding 3×10^8 cells to the treated preparation did not give a 10-fold increase in incorporation over the control rate. Also shown in table I is the fact that neither Zephiran chloride (1: 10,000) or penicillin (10^{-3} M) had much effect on either colony counts or rate of incorporation by the added bacteria. Although not shown here, neither chemical had any effect on incorporation by chloroplast preparations. Finally, anaerobiosis clearly inhibits incorporation by the added bacteria.

In criticism of the above experiments, it is clear the quality of the microbial population grown on nutrient agar is not necessarily identical with that found on the leaf, and for the same reason colony counts on nutrient agar do not necessarily reflect the maximum amount of contamination actually present in the chloroplast preparation. However, as shown below, after removal of the chloroplast RNA with deoxycholate, the remaining amount of pellet RNA agrees reasonably well with the expected pellet RNA content as calculated from plate colony counts and RNA per bacterial cell.

Although microbial contamination appears responsible for much of the incorporation by our chloroplast preparations, the possibility remains that a small rate of incorporation by the chloroplasts was present but masked by the much larger bacterial incorporation. It should be pointed out that the hypochlorite washing of the leaves eliminated about 97% of the observed amino acid incorporation; any activity by the chloroplasts would thus be at most a very minor phenomenon amounting to incorporation of 0.1 mμmole of leucine per milligram of protein. To investigate this possibility, a chloroplast preparation was labeled, washed, and placed on a 0.7 M to 2.0 M linear sucrose gradient (3). Figure 1 shows most of the incorporated C^{14} leucine is found at the bottom of the tube and a smaller peak of radioactivity is found in the chlorophyll region. The exact location of this radioactive peak varied in different experiments from directly under the chlorophyll peak to a little ahead of the chlorophyll. In a companion experiment, bacterial cells were labeled by allowing them to incorporate radioactive amino acids, and then mixed with an unlabeled chloroplast preparation. Figure 2 shows that density gradient centrifugation of this mixture gives about the same profile of radioactivity as in the previous experiment—a large fraction of the cells sediment to the bottom of the tube, but a considerable amount of the radioactivity is still associated with the chlorophyll peak. Thus it is obviously unwarranted to assume that radioactivity in the chlorophyll region in centrifugation experiments of this nature necessarily indicates amino acid incorporation by the chloroplasts.

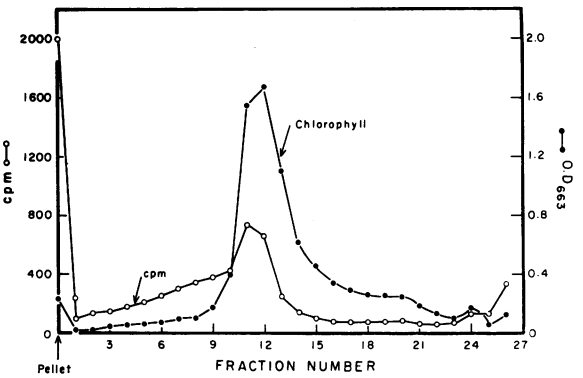

FIG. 1. Sucrose gradient analysis of labeled chloroplast preparation. A spinach chloroplast preparation was labeled for 2.5 hours at 20° with C^{14}-leucine using essentially the same reaction mixture without cold leucine as described under table II. Chloroplasts were washed 2 times with 0.5 M sucrose, 0.02 Tris pH 7.6, 0.01 $MgCl_2$, and 1000 μg/ml leucine, then 0.93 mg chlorophyll in 2.0 ml of sucrose-Tris-Mg was layered on top of 24 ml 0.7 M to 2.0 M sucrose linear gradient (3). Tubes were spun for 40 minutes at 5000 rpm in a Servall HB-4 swinging bucket rotor. Fractions were collected by puncturing the bottom of the tube with a #22 gauge needle. Chlorophyll was estimated by optical density measurement at 663 mμ. Radioactivity was determined by plating 0.1 ml aliquots on filter paper disks and counting on a Nuclear Chicago Model 186. Approximately 100% of the counts layered on the gradient were recovered in the various fractions.

We have previously reported that deoxycholate extraction removes ribosomes from isolated chloroplasts (1). To see whether this observation also is due to bacterial contamination, a mixture of bacteria from the nutrient agar plates were extracted with deoxycholate, then centrifuged at $20{,}200 \times g$. No RNA was found in the supernatant fraction in these experiments; thus it seems that the observation of the existence of chloroplast ribosomes is not due to bacterial contamination. Also Lyttleton (5) could extract ribosomes simply by osmotically shocking spinach chloroplasts in water; an observation that

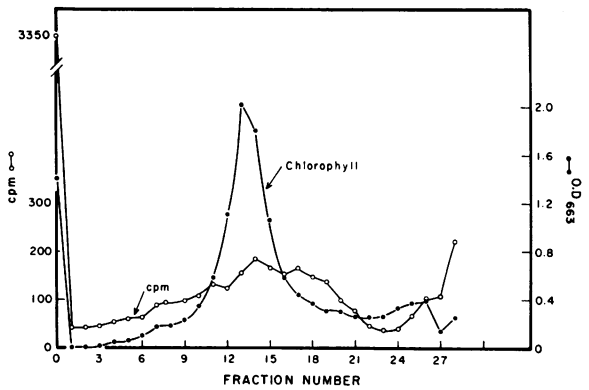

FIG. 2. Sucrose gradient analysis of a mixture of labeled bacteria and unlabeled chloroplasts. Microbial contamination from spinach chloroplast inoculum was cultured on nutrient agar, harvested, and washed 2 times with sucrose-Tris-$MgCl_2$, labeled with C^{14} leucine at 20° for 3 hours, washed 2 times with sucrose-Tris-Mg and 1000 μg/ml cold leucine, then mixed with unlabeled chloroplasts in sucrose-Tris-$MgCl_2$. Two milliliters of this mixture containing 1.16 mg chlorophyll and labeled bacteria were layered on a 0.7 M to 2.0 M sucrose gradient and treated as described under figure 1. Approximately 85 % of the original counts layered were recovered in the various fractions.

we have recently been able to repeat. Finally, adding bacteria with a known and significant RNA content to the chloroplast preparations did not result in any increase in the yield of RNA extractable from the chloroplast fraction with deoxycholate.

Discussion

The results presented here clearly show that most if not all of the amino acid incorporation so easily observable with isolated chloroplasts can be due to contaminating bacteria. In view of this, brief reports of the incorporation of amino acids by chloroplasts without extensive accompanying data showing that bacteria are absent or inactive cannot be accepted without question (1, 2, 4, 7, 8). Previous work on the activity of ribosomes isolated from chloroplasts are on a much sounder basis because conditions used to extract ribosomes from chloroplasts do not solubilize the bacterial ribosomes (1, 7, 8).

The most important and earliest paper concerning protein synthesis by isolated chloroplasts is that of Stephenson, Thimann, and Zamecnik (9). Their chloroplast fraction from tobacco leaf homogenates apparently contained only on the order of 10^5 bacteria, or very much less than we have been afflicted with, and the rates of amino acid incorporation observed by them (about 0.8 mμmole leucine/mg protein hour) were about one third as high as those seen here. A further important control of Stephenson et al. was the complete inhibition of bacterial colony formation from reaction mixtures previously treated with Zephiran, even though Zephiran had no effect on the protein formation.

In our present experiments the results with Zephiran were different in that it neither inhibited the subsequent growth of bacterial colonies nor interferred with protein synthesis. On the other hand the agents which did inhibit protein synthesis for us (chloramphenicol, brief heating, streptomycin, arsenate, puromycin, hypochlorite treatment of the leaves) are just the same ones that inhibit bacterial colony formation.

A most important difference between the results of Stephenson et al. and ours is in the nature of the light effect. In our case light stimulates amino acid incorporation only under relatively anaerobic conditions, and we have been able to show rather clearly that the action of light is simply to produce a bit of O_2 which in turn is needed for oxidative metabolism by the bacteria. In contrast, Stephenson et al. found the light stimulation to occur if anything more clearly under 95 % O_2 than anaerobically. Furthermore their reaction mixtures often contained 0.01 M ascorbate, in which case illumination should have led to oxygen uptake rather than O_2 production. However, the nature of the light stimulation in their case was not explored.

A final point arguing against bacterial activity in the experiments of Stephenson et al. was the shape of the time course for incorporation. The chloroplast fraction activity always sloped off after either 30 or 60 minutes, something which would not be expected from active bacteria. Their observations were terminated at the end of 1 hour, and in no case did it appear that the chloroplasts had completely lost all activity. In our experience the time course curve has been rather variable. In a fair number of experiments we saw a rapid rate for the first hour approximately, then a sloping off for another 1 to 3 hours, and finally a resumption of activity at a much faster pace. We are not able to explain the plateau period but think it might relate to aspects of the physiology of the bacteria or cross-feeding between the chloroplasts and the bacteria. Incorporation of amino acids during the first hour of our experiments showed basically the same characteristics as those seen during the longer time courses with the exception of lesser inhibition by respiratory poisons such as cyanide, azide, or arsenate.

The discrepancies between the experiments of Stephenson et al. and our own are sufficiently great so that it seems quite possible that bacterial contamination was not involved in the results that they observed. However, their system and ours share a number of characteristics which are much easier to explain as being due to bacteria than as the result of chloroplast enzymes: A) O_2 is clearly required for amino acid incorporation, even though chloroplasts have been shown not to be capable of simple oxidative phosphorylation; B) very high concentrations of leucine (0.04 M) are needed for optimal activity; C) no requirement can be shown for added ATP or an ATP generating system: D) ribonuclease does not cause any inhibition at all; E) it is not necessary to

add amino acids other than the one labeled component in order to obtain maximal rates of incorporation.

Further support for the pessimistic point of view may be found in recent experiments of S. G. Wildman and D. Spencer (personal communication). Using a much more sophisticated procedure for isolating tobacco chloroplasts, they have apparently been able to demonstrate an ATP requiring, O_2 independent, ribonuclease sensitive amino acid incorporation by the chloroplast fraction. If this is truly the nature of the chloroplast protein forming system, then results such as those above are more certainly questionable ones.

There are probably 2 reasons for the severity of the bacterial contamination in spinach chloroplast preparations. In the first place the leaves were purchased from local markets. They undoubtedly had spent a number of days in storage during which time bacterial growth would be favored by the presence of any damaged tissues. When working with leaves of spinach, bean, or pea grown in the greenhouse and used immediately after harvesting, lower initial rates of incorporation were always seen. In the second place the conditions used in centrifuging chloroplasts ($2000 \times g$ for 7 minutes) are sufficient to sediment a very large fraction of the accompanying bacteria. If by any chance the buffer solutions themselves are contaminated with bacteria every time a chloroplast preparation is washed in fresh buffer more bacteria will be added to the preparation.

It should be noted that the preparative conditions shown here to result in chloroplasts highly contaminated with bacteria are essentially identical to those usually employed in studies of chloroplast biochemistry, photosynthetic or otherwise.

Summary

Apparent incorporation of amino acids into protein by isolated spinach (*Spinacia oleracea*) chloroplasts was found to be due to contaminating bacteria which are sedimented together with the chloroplasts during their initial isolation. At least 75 % of the labeled protein is separable from chloroplasts of a labeled preparation in a subsequent density gradient centrifugation, and the conditions which inhibit incorporation (brief heating at 50°, hypochlorite treatment of the leaf surface before homogenizing, or addition of streptomycin, chloramphenicol, or puromycin) are those which inhibit growth of the contaminating bacteria.

Previous reports of stimulation of incorporation by light are confirmed, but in our case the enhancement appears to be due to evolution of a small amount of O_2 by the chloroplasts. This O_2 in turn stimulates the metabolism of the highly aerobic contaminating bacteria under conditions of low O_2 tension. Reagents previously reported not to inhibit protein synthesis by chloroplasts (penicillin, Zephiran) are shown not to inhibit growth of contaminating bacteria in the present instance. It is concluded that certainly not in the present experiments, and possibly not in others reported to date has it been demonstrated unequivocally that isolated chloroplasts are capable of amino acid incorporation. On the other hand the present results do not cast doubt on experiments with isolated chloroplast ribosomes, because bacterial ribosomes are not extracted by the procedure used to isolate those from chloroplasts.

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Literature Cited

1. App, A. and A. T. Jagendorf. 1963. Incorporation of labeled amino acids by chloroplast ribosomes. Biochim. Biophys. Acta 76: 286–92.
2. App, A. and A. T. Jagendorf. 1963. In vitro incorporation of labeled amino acids by spinach chloroplasts. Plant Physiol. 38: vii.
3. Britten, R. J. and R. B. Roberts. 1960. High-resolution gradient sedimentation analysis. Sci. 131: 32–33.
4. Heber, U. 1962. Protein synthesis in chloroplasts during photosynthesis. Nature 195: 91–92.
5. Lyttleton, J. W. 1961. Isolation of ribosomes from spinach chloroplasts. Exptl. Cell Res. 26: 312–17.
6. Mans, R. F. and G. D. Novelli. 1960. A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein. Biochem. Biophys. Res. Commun. 3: 540–43.
7. Parthier, B. and R. Wollgiehn. 1963. Zur Frage der Amino Saure-Inkorporation in RNS-reiche Partikeln aus Chloroplasten. Naturwissenschaften 50: 598–99.
8. Sissakian, N. M., I. I. Filippovich, E. N. Suetailo. 1962. Participation of chloroplast ribosomes in protein synthesis. Dokl. Akad. Nauk. SSSR 147: 488–89.
9. Stephenson, M. L., K. V. Thimann, and P. C. Zamecnik. 1956. Incorporation of C^{14} amino acids into proteins of leaf disks and cell free fractions of tobacco leaves. Arch. Biochem. Biophys. 65: 194–209.