in the lecithin makes the high ratio complexes possible. On the other hand cardiolipin, inositide phospholipid, and the three forms of phosphatidylethanolamine prepared by the two methods show similar complex-forming properties as the fractions of this type reported previously.

The complex prepared with cardiolipin is similar to a complex recently reported by Green and Fleischer (1963). The high ratio (100:1) phosphatidylethanolamine complex which they report may be related to the phosphatidylethanolamine complex which we obtain with the low-iodine value phosphatidylethanolamine fractions. This high ratio type of complex may also be related to the complex which we obtained using the fully saturated dimyristoylethanolamine phospholipid which had a ratio of 100:1 (Das *et al.*, 1962).

Acknowledgment

We wish to thank Dr. G. Rouser for his generous advice and guidance in purification of the phospholipids by the DEAE-cellulose system. Mr. D. Myers provided excellent assistance.

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Protein Synthesis by Cell-free Extracts from Tobacco Leaves. I. Amino Acid Incorporating Activity of Chloroplasts in Relation to Their Structure*

R. I. B. Francki,† N. K. Boardman,‡ and S. G. Wildman

ABSTRACT: The amino acid incorporating activity of isolated tobacco leaf chloroplasts was studied in relation to the biphasic structure of the chloroplast. Active chloroplasts were prepared by using extraction media with appropriate osmotic properties. Over 80% of the amino acid incorporating activity of the chloroplasts could be transferred into 17,000 *g* supernatants by washing the chloroplasts in a buffer of low molarity. Microscopic observations indicated that removal of incorporating activity correlated with the loss of the mobile phase (stroma) of the chloroplast. It is concluded that the materials responsible for *in vitro* protein synthesis by chloroplasts including s-RNA and activating enzymes are located in the mobile phase of the chloroplast.

In a recent communication (Spencer and Wildman, 1964) a method was described for obtaining a cell-free system from tobacco leaves that incorporated amino acids into protein. The incorporating activity was dependent on an ATP[1] generating system, Mg^{2+}, and a mixture of amino acids. The activity was destroyed by RNAase, puromycin, and chloromycetin. Phenylalanine incorporation was specifically stimulated by poly-U. Thus the properties of the leaf system corresponded with ribosome preparations obtained from such organisms as *Escherichia coli* (Nirenberg and Matthaei, 1961), *Chlamydomonas* (Sager *et al.*, 1963), reticulocytes (Schweet *et al.*, 1958), and the like. What was different about the leaf system was that over 80% of the activity in a cell-free homogenate could be removed as a pellet

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[1] Abbreviations used in this work: ATP, adenosine-5′-triphosphate; RNAase, ribonuclease; UTP, CTP, and GTP, uridine-, cytidine-, and guanosine-5′-triphosphates, respectively.

after centrifuging for 10 minutes at 1000 *g*. The pellet consisted mainly of nuclei and chloroplasts, and the activity appeared to be confined to the chloroplasts.

Extensive observations of chloroplasts in living mesophyll cells of leaves by phase and fluorescence microscopy have shown the chloroplast to consist of two components, a *stationary component* and a *mobile phase*, the latter having been previously called the chloroplast jacket (Wildman *et al.*, 1962; Spencer and Wildman, 1962; Hongladarom, 1964). The stationary component consists of the lamellar system together with stacks of grana, chlorophyll being confined to the grana. The mobile phase appears to surround and penetrate throughout the stationary component. Isolated chloroplasts differ in appearance from the chloroplasts in the living cell, but by the use of special media which facilitate preservation of the microscopic appearance of organelle structures in homogenates (Hongladarom, 1964) it is possible to recognize when isolated chloroplasts still possess the mobile phase.

In the studies described in this communication, isolated chloroplasts were examined microscopically and assayed for their amino acid incorporating activity to determine whether the protein synthesizing activity of the chloroplast is an integral part of the stationary component or is located in the mobile phase of the chloroplasts. The accompanying communication (Boardman *et al.*, 1965) describes the physical characteristics of the system which is responsible for the activity.

Materials and Methods

Preparation of Tobacco Leaf Chloroplasts. Chloroplasts were prepared from leaves of young plants (*Nicotiana tabacum* var. Turkish Samsun) by the methods already described (Spencer and Wildman, 1964). Fresh weight 5-g samples of deribbed leaf fragments were chopped into a fine mince with 6 ml of extracting medium. The resulting brei was filtered through three layers of fine cloth to remove unbroken cells and the filtrate was centrifuged at 1000 *g* for 10 minutes. The supernatant was discarded and the pellets were resuspended. Material pelleted from 5 g of leaf tissue was resuspended in 3.5 ml of medium. Several different media were used for extraction and for the resuspension of 1000 *g* pellets: (1) *Honda medium*, 2.5% Ficoll, 5% dextran, 0.25 M sucrose, 0.025 M Tris at *p*H 7.8, 1 mM magnesium acetate, and 4 mM mercaptoethanol; (2) NaCl *medium*, 0.35 M NaCl, 0.025 M Tris at *p*H 7.8, 1 mM magnesium acetate, and 4 mM mercaptoethanol; (3) *sucrose medium*, 0.5 M sucrose, 0.025 M Tris at *p*H 7.8, 1 mM magnesium acetate, and 4 mM mercaptoethanol; (4) *Tris*-Mg-SH *medium*, 0.01 M Tris at *p*H 7.8, 5 mM magnesium acetate, and 3 mM mercaptoethanol. Unless otherwise stated, leaves were extracted in Honda medium and the 1000 *g* pellets were resuspended in Tris-Mg-SH medium. Microscopic examination of these suspensions showed them to contain chloroplasts, nuclei, and starch grains. It has already been shown by Spencer and Wildman (1964) that negligible amino acid incorporating activity was associated with nuclei and this has been confirmed in the present study. Therefore in this communication these suspensions will be referred to as *Chloroplasts*. In some experiments *Chloroplasts* were further fractionated by centrifugation at 17,000 *g* for 15 minutes into a *Chloroplast supernatant* and a pellet which was resuspended in the original volume of medium. The resuspended pellet will be referred to as *Chloroplast pellet*. By repeating the procedure on the *Chloroplast pellet*, a *Washed chloroplast pellet* was obtained.

Standard Assay of Amino Acid Incorporation. Methods for the assay of amino acid incorporation were similar to those described by Spencer and Wildman (1964).

To 0.4 ml. of *Chloroplasts* was added 0.105 ml of a mixture containing Tris, *p*H 7.8 (2.3 μmoles), magnesium acetate (2.3 μmoles), KCl (13.8 μmoles), mercaptoethanol (1.3 μmoles), ATP (0.2 μmole), phosphoenolpyruvate (1.25 μmoles), pyruvate kinase (10 μg), UTP (0.01 μmole), CTP (0.01 μmole), GTP (0.01 μmole), and a mixture of 0.0125 μmole each of nineteen amino acids (omitting valine). A 0.5 mM solution (5 μl) of [^{14}C]valine (specific activity 200) was added, and incubation was carried out for 30 minutes at 25°.

Unless otherwise stated, all radioactivity measurements reported in this paper refer to the radioactivity remaining in the precipitate after extraction for 20 minutes with hot trichloroacetic acid at 80°. Precipitates were placed on Millipore filters and counted in a Nuclear Chicago gas-flow counter fitted with a Micromil window (counting efficiency, approximately 30%).

Preparation of Chloroplast High-Speed Supernatant. Chloroplasts were isolated by grinding 50 g fresh weight of deribbed tobacco leaves in 100 ml of Honda medium, first in a Waring Blendor for 30 seconds at 50% of the line voltage and then in a mortar with a pestle. All operations were carried out at 0–4°. Chloroplasts were isolated and suspended in 20 ml Tris-Mg-SH medium. This suspension was centrifuged for 2 hours at 144,000 *g* and yielded a supernatant solution which was stored at −70°. Before use, the high-speed supernatant was thawed and dialyzed against Tris-Mg-SH medium for 2 hours at 0–4°. Under standard assay conditions, 0.2-ml aliquots of *High-Speed Supernatant* incorporated 100–300 cpm into a cold trichloroacetic acid–insoluble product. This product was completely soluble in hot trichloroacetic acid.

Microscopy and Special Reagents. Observations were made at magnifications of 1250 with a Zeiss Research Model microscope equipped with Neofluar phase-contrast objectives and a V Z condenser. The sources of special reagents were those listed by Spencer and Wildman (1964).

Experimental Results

Amino Acid Incorporation by Chloroplasts. Extending the work of Spencer and Wildman (1964), the results summarized in Table I indicate that the amino acid incorporating activity of *Chloroplasts* is dependent on the presence of mercaptoethanol in the suspending medium, but the concentration does not appear to be critical over a relatively wide range. High concentra-

TABLE I: Requirements of Mercaptoethanol for Amino Acid Incorporation by *Chloroplasts* from Tobacco Leaves.[a]

Concentration of Mercaptoethanol (M)	[^{14}C]Valine Incorporation[b]
Nil	129
2.6×10^{-4}	716
7.7×10^{-4}	516
2.6×10^{-3}	581
7.7×10^{-3}	625
2.6×10^{-2}	547

[a] Chloroplasts were prepared in *Honda* medium and resuspended in *Tris*-Mg-SH medium containing various concentrations of mercaptoethanol. [b] Hot trichloroacetic acid–insoluble counts per minute per assay.

tions of Mg^{2+} are inhibitory to the system and incorporation is nearly completely arrested at a concentration of 2×10^{-1} M (Table II).

It has been our experience that *Chloroplasts* from tobacco leaves vary in their activity from experiment to experiment. We have not made a systematic study of the effects of leaf or plant age on the activity of *Chloroplasts*, but our most active preparations have been made from rapidly expanding leaves (5–7 cm in length) of rapidly growing young plants (about 7–8 cm in height). It was also found that addition of phosphoenolpyruvate to the incubation mixture as the tricyclohexylammonium salt produced the same activity as when the sodium salt was used.

TABLE II: Effect of Mg^{2+} Concentration on Amino Acid Incorporation by *Chloroplasts* from Tobacco Leaves.[a]

Concentration of Magnesium Acetate (M)	[^{14}C]Valine Incorporation[b]
Nil	1119
2×10^{-3}	1329
4.6×10^{-3}	1275
2×10^{-2}	390
4.6×10^{-2}	145
2×10^{-1}	20

[a] Chloroplasts were prepared in *Honda* medium and resuspended in *Tris*-Mg-SH medium containing various concentrations of Mg^{2+}. [b] Same as Table I.

Activity of Chloroplasts in Relation to the Presence of the Mobile Phase. The mobile phase seen on chloroplasts contained in living cells (Figure 1) can also be

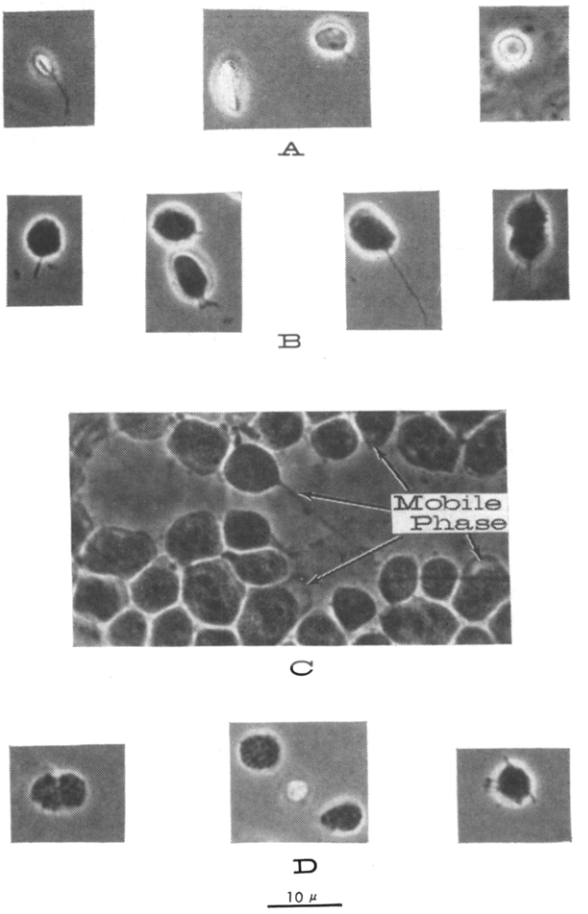

FIGURE 1: Appearance of mobile phase on chloroplasts in living tobacco leaf cells and isolated chloroplasts. Part (C) is a portion of a typical, living tobacco leaf cell exposed for phase photomicrography by free-hand sectioning of leaf tissue. The mobile phase is apparent on nearly every chloroplast. The grana of the stationary component are also conspicuous. The larger, dark objects in the stationary component are starch grains. The dynamic nature of the mobile phase can be seen on a cine-micro photographic film: *Appearance and Behavior of Organelles in Living Plant Cells* (2nd edition), which is available for loan from Educational Film Sales, Extension Division, University of California, Berkeley 4, Calif. Chloroplasts isolated in *Honda* medium have the appearance shown in (A), (B), and (D), together with about 10% of the chloroplasts which are swollen and disorganized (not illustrated). About 70% of the isolated chloroplasts have the appearance of (A) and (B): either tightly folded (A) or partially folded to unfolded as in (B). Both (A) and (B) chloroplasts have retained the mobile phase, and its presence in (B) closely resembles that seen on chloroplasts in living cells, even to the extent of conspicuous protuberances. About 20% of the chloroplasts appear as in (D): grana are conspicuous but the mobile phase is greatly reduced in extent. In the lower left corner, two stationary components are enclosed by a thin film of mobile phase. (Photomicrographs by courtesy of Dr. Tasani Hongladarom.)

identified in isolated chloroplasts (Hongladarom, 1964). The retention of the mobile phase by chloroplasts depends primarily on the osmotic pressure of the media used for their isolation, and either sucrose or NaCl is suitable. The addition of Ficoll to the sucrose causes the mobile phase to have a more relaxed appearance such that the irregular outlines seen in the living cell are retained together with protuberances of the mobile phase extending out from the surface of many chloroplasts. Under hypotonic conditions the mobile phase is dispersed and the stationary component swells to a marked extent. Starch grains are released from the stationary component and appear as free objects in the homogenate. In the absence of Ficoll, sucrose is a better preservative of the mobile phase than is NaCl; chloroplasts will stand several cycles of washing in sucrose and still retain some of the mobile phase, whereas one wash in NaCl generally results in the nearly complete loss of the mobile phase without concomitant swelling of the lamellar system. We have investigated whether the presence or absence of the mobile phase is correlated with the presence or absence of the amino acid incorporating activity.

Spencer and Wildman (1964) used *Honda* medium containing sucrose and Ficoll for preparation of cell-free extracts of tobacco leaves, and resuspended the *Chloroplasts* in the same medium. As seen from the data in Table III, *Chloroplasts* prepared in 0.5 M sucrose

TABLE III: Comparison of Amino Acid Incorporating Activity by *Chloroplasts* from Tobacco Leaves Extracted in Various Media.

Extraction Medium[a]	[^{14}C]Valine Incorporation[b] Expt 1	Expt 2
Honda	802	1142
0.35 M NaCl	850	1580
0.50 M Sucrose	774	
Tris-Mg-SH	408	225

[a] *Chloroplasts* were extracted in the media listed and resuspended in *Tris*-Mg-SH medium. [b] Hot trichloroacetic acid–insoluble counts per minute per assay corrected for slight differences in chlorophyll contents of extracts.

or 0.35 M NaCl are as active in amino acid incorporation as those prepared in *Honda* medium, whereas those isolated in a buffer of low molarity have much lower activities, showing that retention of the protein-synthesizing activity of *Chloroplasts* depends primarily on the osmotic pressure of the isolation medium.

We have found also that the amino acid incorporating activity of *Chloroplasts* is affected by the medium in which they are resuspended (Table IV). It appears that, of the media tested, *Tris*-Mg-SH medium is the most favorable. The marked inhibition of the incorporation of [^{14}C]valine by resuspending the *Chloroplasts* in 0.35 M NaCl is the result of direct inactivation by the salt of the system responsible for incorporation. In a typical experiment the addition of 0.35 M NaCl to *Chloroplasts* in *Tris*-Mg-SH medium reduced the incorporation of [^{14}C]valine from 730 to 210 cpm. The fact that appropriate osmotic conditions were essential for the retention of amino acid incorporating activity by *Chloroplasts*, but were not required for the activity itself, suggested that the activity was being released from *Chloroplasts* when they were resuspended in a buffer of low molarity. Separation of *Chloroplasts* in *Tris*-Mg-SH medium into a *Chloroplast supernatant* and a *Chloroplast pellet* by centrifugation at 17,000 *g* for 15 minutes showed that the activity of the clear yellow supernatant exceeded that of the chlorophyll-containing

TABLE IV: Effect of Various Suspending Media on Amino Acid Incorporating Activity by *Chloroplasts*.

Suspension Medium[a]	[^{14}C]Valine Incorporation[b] Expt 1	Expt 2
Honda	624	731
0.35 M NaCl	39	299
0.50 M Sucrose	420	591
Tris-Mg-SH	721	1028

[a] *Chloroplasts* were extracted in *Honda* medium and resuspended in the media listed above. [b] Same as Table I.

pellet. In ten such experiments, the average ratio of activity of *Chloroplast supernatant* to *Chloroplast pellet* was 1.53. In contrast, when *Chloroplasts* were resuspended in *Honda* medium, the activity of the *Chloroplast pellet* exceeded that of the *Chloroplast supernatant*. In six experiments, the mean ratio of *Chloroplast supernatant* to *pellet* was 0.42. Microscopic examination of *Chloroplasts* resuspended in *Honda* medium showed many of them to have retained the mobile phase (Figure 2). A large portion of the population either was in the folded condition or possessed the irregular outlines and protuberances characteristic of the mobile phase. In contrast, *Chloroplasts* resuspended in *Tris*-Mg-SH medium were unfolded and the stationary components were somewhat swollen. The mobile phase could not be detected on most of the chloroplasts in the population.

Data from two typical experiments where both *Tris*-Mg-SH and *Honda* media were used for resuspending *Chloroplasts* are summarized in Table V. The activity of the *Chloroplast pellet* obtained from *Chloroplasts* suspended in *Tris*-Mg-SH medium was stimulated

TABLE V: Distribution of Protein Synthesizing Activity in *Chloroplast supernatant* and *Chloroplast pellet*.

Fraction[a]	[^{14}C]Valine Incorporation[b]			
	Expt 1		Expt 2	
	Tris-Mg-SH Medium	Tris-Mg-SH Medium + HSS[c]	Honda Medium	Honda Medium + HSS[c]
Chloroplasts	1036	1251	305	303
Chloroplast supernatant	465	480	148	118
Chloroplast pellet	240	423	415	496
Chloroplast supernatant + *Chloroplast pellet*	705	903	563	614
$\frac{\text{Chloroplast } s}{\text{Chloroplast } p}$	1.94	1.13	0.36	0.24

[a] *Chloroplasts* were extracted from 10 g of leaf tissue with *Honda* medium and after pelleting were resuspended in 7 ml of *Tris*-Mg-SH medium (expt 1) or 7 ml of *Honda* medium (expt 2). After 10 minutes a 3-ml sample of the *Chloroplasts* was centrifuged for 15 minutes at 17,000 *g*. In each experiment the *Chloroplast pellet* was resuspended in 3 ml of *Tris*-Mg-SH medium. [b] Same as Table I. [c] HSS = *High-Speed Supernatant*. A *High-Speed Supernatant* preparation (0.2 ml) was added to each assay.

TABLE VI: Effect of Dialysis on Distribution of Protein Synthesizing Activity in *Chloroplast supernatant* and *Chloroplast pellet*.

Fraction[a]	[^{14}C]Valine Incorporation[b]			
	Tris-Mg-SH Medium		Honda Medium	
	Non-dialyzed	Dialyzed	Non-dialyzed	Dialyzed
Chloroplast supernatant	276	387	149	188
Chloroplast pellet	337	301	648	454
$\frac{\text{Chloroplast supernatant}}{\text{Chloroplast pellet}}$	0.82	0.92	0.23	0.41

[a] Fractionating procedures as in Table V. [b] Same as Table I.

markedly by the addition of *High-Speed Supernatant*, resulting in a lowering in the ratio of the activity of the *Chloroplast supernatant* to pellet. In five experiments in which *High-Speed Supernatant* was added, the average ratio was 1.00. The *Chloroplast pellet* from *Chloroplasts* prepared in Honda medium was less dependent on *High-Speed Supernatant*, suggesting that the s-RNA and/or activating enzymes are located in the mobile phase of the chloroplast.

That the differences in distribution of activity between *Chloroplast supernatant* and *Chloroplast pellet* are not a reflection of a difference in amino acid pool of chloroplasts resuspended in *Honda* medium, compared with those resuspended in *Tris*-Mg-SH medium, is shown by the data given in Table VI. Samples of *Chloroplast supernatant* and *Chloroplast pellet*, prepared from both *Chloroplasts* resuspended in *Honda* medium and *Chloroplasts* resuspended in *Tris*-Mg-SH medium, were dialyzed against *Tris*-Mg-SH medium for 3 hours, with shaking and with changes of medium at hourly intervals. The dialyzed samples were compared with undialyzed aliquots for their amino acid incorporating activity. The activities did not change appreciably as the result of dialysis.

The results given in Table IV confirm the finding of Spencer and Wildman (1964) that the incorporating activity of the 1000 *g* pellet (*Chloroplasts*) was not associated with nuclei. Although the washed pellet obtained after disruption of *Chloroplasts* with Triton X-100 still contained intact nuclei, as observed microscopically, its amino acid incorporating activity was less than 2% of the activity of *Chloroplasts*.

Since the previous experiments showed that the chlorophyll-containing pellet still retained appreciable activity even when resuspended in *Tris*-Mg-SH medium, it was of interest to see whether this residual activity could be removed by further washing with *Tris*-Mg-SH medium. The results presented in Figure 3 show

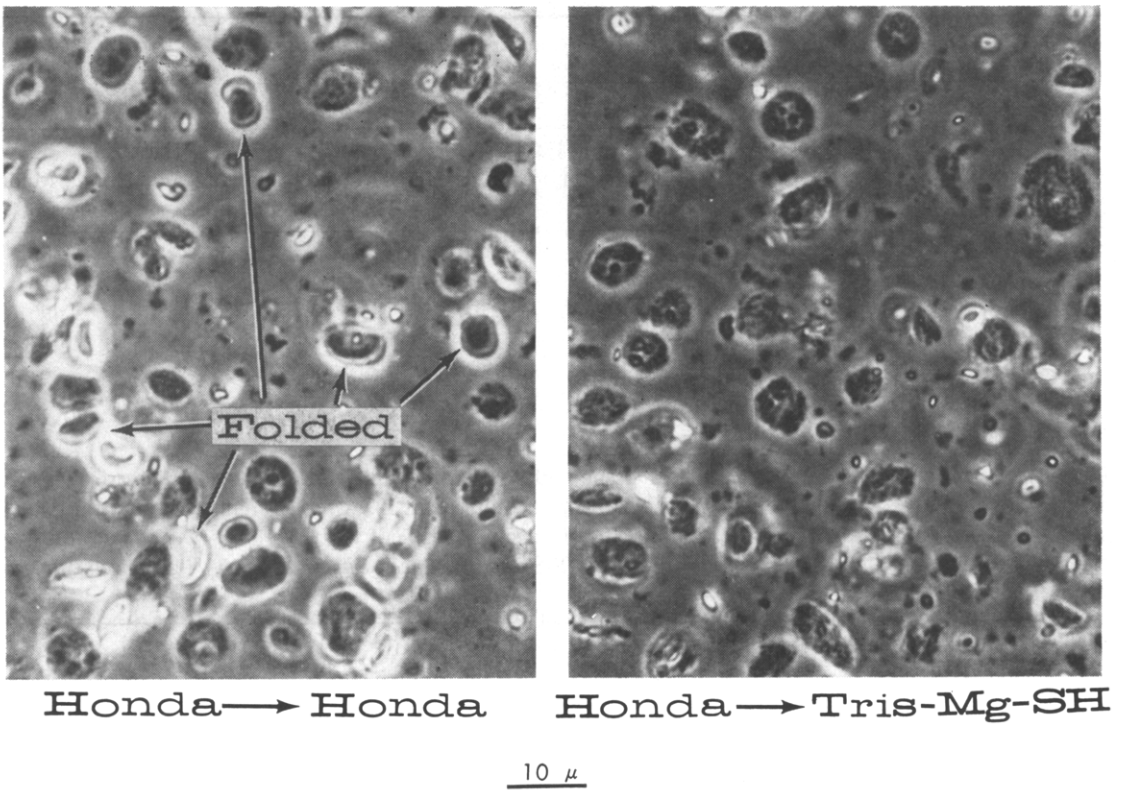

FIGURE 2: Comparison of the microscopic appearance of *Chloroplasts* after resuspension in *Honda* or *Tris*-Mg-SH medium. *Chloroplasts* were extracted in *Honda* medium, centrifuged into pellets, and resuspended into media shown. The photomicrographs do not satisfactorily show the large number of starch grains expelled from the chloroplasts into the medium when *Chloroplasts* are caused to unfold and lose the mobile phase by resuspension into *Tris*-Mg-SH medium. (Photomicrographs by courtesy of Dr. Tasani Hongladarom.)

that the *Chloroplast pellet* became progressively less active with washing, whereas a large proportion of the activity lost from the *Chloroplast pellet* was recovered in the 17,000 *g* supernatant. The small residual activity remaining after the fourth wash was rendered nonsedimentable at 17,000 *g* by treatment of the pellet with 1% Triton X-100 as shown in Figure 3. Data in Table VII show that this neutral detergent, which completely disrupts chloroplasts, has very little inhibitory effect on the amino acid incorporating activity. At a concentration of 4%, the detergent solubilizes chlorophyll to the extent that it is nonsedimentable at centrifugal forces as high as 100,000 *g* for 2 hours.

TABLE VII: Protein-synthesizing Activity of *Chloroplasts* after Treating with Triton X-100.

Fraction	[^{14}C]Valine Incorporation[a]	
	Control Chloroplasts[b]	Triton X-100–treated Chloroplasts[c]
Chloroplasts	305	259
Chloroplast supernatant	167	245
Chloroplast pellet	70	22
Washed Chloroplast pellet	60	4

[a] Same as Table I. [b] See methods for fractionation procedure. [c] *Chloroplasts* obtained from 5 g of leaf tissue were suspended in 1 ml *Tris*-Mg-SH medium at 0° and 0.25 ml 20% Triton X-100 was added. After 10 minutes the preparation was made up to a total volume of 3.5 ml with the same medium. *Chloroplast pellet* was washed with 3.5 ml of *Tris*-Mg-SH medium without the detergent.

Discussion

A concept of the biphasic organization of a higher plant, grana-containing chloroplast, is shown in Figure 4. The chloroplast complex consists of a stationary component and a mobile phase. In the living cell, the mobile phase of the chloroplast continually changes shape and its extent. The stationary component is a system of elliptical bags stacked on top of each other to produce the appearance of a smooth outline of the sta-

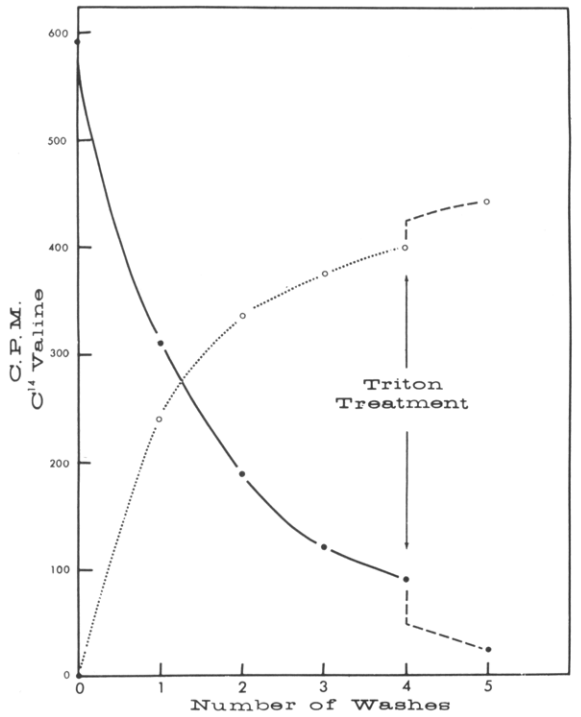

FIGURE 3: Effect of successively washing *Chloroplasts* with *Tris*-Mg-SH medium on their incorporation of [^{14}C]valine. Solid line, incorporation by *Chloroplasts*. Dotted line, sum of the incorporation activities of the successive washes. Broken line, after Triton X-100 treatment. A *High-Speed Supernatant* (0.2 ml) preparation was added to all assays for incorporation.

and the osmotic concentration is lowered gradually by allowing water to diffuse beneath the cover slip, the mobile phase may be seen to swell into a balloon which breaks with the dispersal of its contents before swelling of the stationary component ensues. Even after swelling to a marked extent, the structure of the stationary component is still recognizable and fluorescence microscopy shows that chlorophyll is still retained within the swollen structure.

Thus there is a striking correlation between the loss of mobile phase as viewed microscopically and the transfer of the incorporating activity from the *Chloroplasts* into the *Chloroplast supernatant* solution by removal of the osmotic protection. After transfer has occurred the chlorophyll-containing stationary component remains sufficiently intact to sediment as a pellet, but the mobile phase is no longer recognizable as part of the chloroplast complex. Thus the conclusion is reached that, of the activity associated with osmotically protected *Chloroplasts*, at least 80% is located in the mobile phase. It is possible that the 20% of residual activity remaining with the stationary component after dispersal of the mobile phase is still in a portion of the mobile phase within the interstices of the lamellar system and less susceptible to dispersal into the supernatant. The experiments also suggest that the amino acid activating enzymes and/or s-RNA necessary for *in vitro* protein synthesis are also located in the mobile phase of chloroplasts. It is only after some of the mobile phase has been removed that the chloroplast becomes dependent upon added sources of s-RNA and/or activating enzymes.

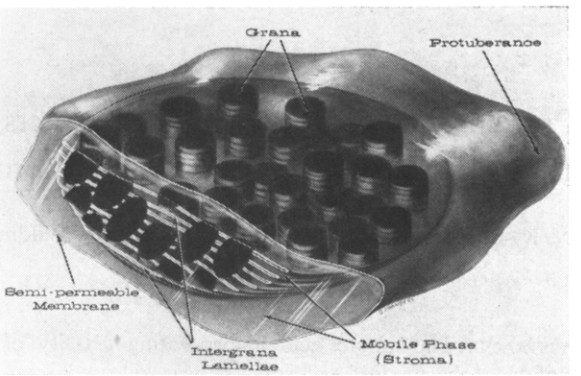

FIGURE 4: A model of the organization of a higher plant, grana-containing chloroplast, to show the relationship between the mobile phase and the chlorophyll-containing stationary component. The chloroplast complex is separated from the cytoplasm by the boundary of the mobile phase.

tionary component. The bags are equivalent to the lamellae seen in cross sections of chloroplasts in the electron microscope. Stacks of laminated grana where chlorophyll is located are interspersed throughout the system of lamellar bags. The mobile phase not only surrounds the stationary component, but also penetrates into the interstices separating the lamellar bags. The outer surface of the mobile phase is the boundary of the chloroplast complex which separates the entire structure from the cytoplasm. Since the mobile phase displays osmotic properties, the outer surface appears to be a limiting, semipermeable membrane. This concept of the chloroplast has emerged from the need to interpret extensive observations of the appearance and behavior of chloroplasts in living cells of spinach, tobacco, and other leaves, of the osmotic behavior of isolated chloroplasts, and of structural aspects of isolated chloroplasts (Spencer and Wildman, 1962; Wildman *et al.*, 1962; Hongladarom, 1964). The proposed model is consistent with observations of sectioned chloroplasts in the electron microscope.

The experiments presented in this paper show that appropriate osmotic conditions are necessary for preserving the association of the amino acid incorporating system with the chloroplasts. When such chloroplasts are kept under continuous microscopic observation,

The question of what materials in the mobile phase are responsible for *in vitro* protein synthesis is the subject of the accompanying communication, where evidence

is presented to show that ribosome monomers are mainly responsible for the incorporating activity of *Chloroplasts*. Unpublished experiments have also shown that two classes of ribosomes, 70 S and 80 S, exist in leaves, in confirmation of previous results by Lyttleton (1962). The two classes of ribosomes are present in about equal amounts. When *Chloroplasts* prepared in *Honda* medium are removed from a leaf homogenate as a 1000 *g* pellet, ultracentrifugal examination of the remaining "cytoplasmic" supernatant shows that over 80% of the cytoplasmic ribosomes are of the 80 S class, in marked contrast to a *Chloroplast supernatant* where over 80% of the ribosomes are of the 70 S class. Thus removal of *Chloroplasts* with mobile phase intact also preferentially removes the 70 S class of ribosomes. Preferential removal of the 70 S ribosomes is most likely the result of the 70 S ribosomes' being contained within the boundaries of the mobile phase during isolation of *Chloroplasts*.

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Protein Synthesis by Cell-free Extracts from Tobacco Leaves. II. Association of Activity with Chloroplast Ribosomes*

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ABSTRACT: The amino acid incorporating activity of isolated tobacco leaf chloroplasts was released from the mobile phase of the chloroplasts and fractionated by centrifugation on density gradients of sucrose. It was found that most of the activity was associated with ribosome monomers.

Analytical ultracentrifugation showed the presence of two classes of ribosomes in the chloroplast extracts with sedimentation coefficients of about 70 S and 80 S. The 70 S ribosomes predominated and accounted for over 80% of the ribosomes of the chloroplast fraction. There was no evidence from these analyses for the presence of substantial amounts of polyribosomes in any of the preparations.

Evidence was presented in the preceding paper (Francki *et al.*, 1965) that most of the activity responsible for amino acid incorporation into protein in isolated tobacco leaf chloroplasts is located in the mobile phase of the chloroplasts. The activity could be separated from the chlorophyll-containing stationary component without drastic alteration in the structure of the stationary component. Protein synthesis was not dependent upon the structural integrity of the chloroplasts since there was no decrease in the incorporating activity after the chloroplasts were solubilized by the nonionic detergent, Triton X-100. Lyttleton (1962) has reported the isolation from spinach chloroplasts of ribosome monomers which were distinguishable from other plant ribosomes by their low sedimentation coefficient of 66 S. We have confirmed this result for tobacco leaf chloroplasts. The question arose, therefore, as to whether the 66 S ribosomes, or some form of polyribosomes such as those identified in *Escherichia coli* (Marks *et al.*, 1962), reticulocytes (Warner *et al.*, 1963), mammalian cells (Gierer, 1963), or plant tissues (Clark *et al.*, 1963) were responsible for amino acid incorporation by chloroplasts. From the results to be presented, it is evident that ribosome monomers released from the mobile phase of chloroplasts account

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