MEMBRANES AND MUTATIONS IN CHLAMYDOMONAS REINHARDI

R. P. LEVINE

The Biological Laboratories
Harvard University
Cambridge, Massachusetts 02138

The molecular structure and mode of assembly of biological membranes are subjects that can be studied with organisms in which membrane structure and function have been altered as a consequence of gene mutation. The investigation of membrane proteins would seem to be particularly amenable to a genetic approach since the proteins are direct products of gene transcripts. There are mutant strains of the unicellular green alga, *Chlamydomonas reinhardi*, that are unable to carry out normal photosynthesis because they lack a chloroplast membrane-bound component of the photosynthetic electron transport chain (LEVINE 1969). The fine structure of these mutant strains has been the subject of several studies (GOODENOUGH and LEVINE 1969, 1970). Several strains have also been examined that are deficient in photosynthetic pigments (GOODENOUGH *et al.* 1969, GOODENOUGH and STAEHELIN 1971), and one of these strains, *ac-5*, is found to have chloroplast membranes with several distinctive features. In this paper I will give a brief description of our experiments with *ac-5* and will present evidence that the *ac-5* mutation has a direct effect on the synthesis of membrane proteins that are intimately involved in chloroplast membrane fusion.

THE FUSION AND STACKING OF CHLOROPLAST MEMBRANES

Cells of *C. reinhardi* possess a single chloroplast (Figure 1) in which there are sac or disc-like membraneous structures called thylakoids (Figure 2). Single thylakoids are found within the chloroplast, but more frequently the thylakoid membranes fuse to form stacks (Figure 2). These stacks correspond to the grana found in the chloroplasts of higher plants. One of the distinctive features noted early in the study of *ac-5* (GOODENOUGH and STAEHELIN 1971) was that the chloroplast membranes did not fuse to form stacks (Figure 3) when the cells were grown mixotrophically (in the light with CO_2 and sodium acetate as carbon sources). This was of interest because it had been hypothesized (HOMANN and SCHMID 1967) that the stacks of membranes were required for various

photosynthetic reactions to occur, particularly those associated with photochemical system II. Since *C. reinhardi* can grow either phototrophically with CO_2 as the sole carbon

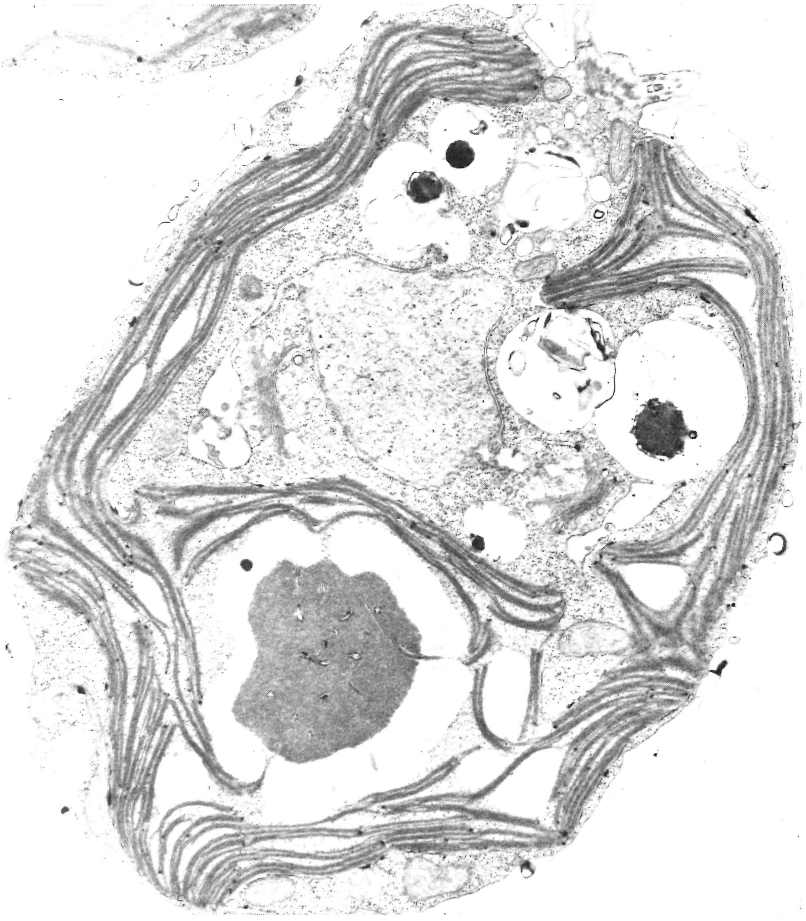


FIGURE 1. Electron micrograph showing the fine-structure of a cell of *C reinhardi*. The large cup-shaped structure is the chloroplast, at the base of which lies a large pyrenoid surrounded by starch. Magnification: 22,200X. This micrograph and the others in this paper were provided by Professor Ursula W. Goodenough.

source or in the presence of acetate as a source of reduced carbon, GOODENOUGH and STAEHELIN next inquired whether *ac-5* was capable of phototrophic growth and thus of carrying out photosynthesis. They found that the mutant strain could indeed grow phototrophically but that its growth rate was

half that of wild-type cells and attributable, at least in part, to its relative pigment deficiency. They noted, however, that the pigment deficiency was the same in cells grown

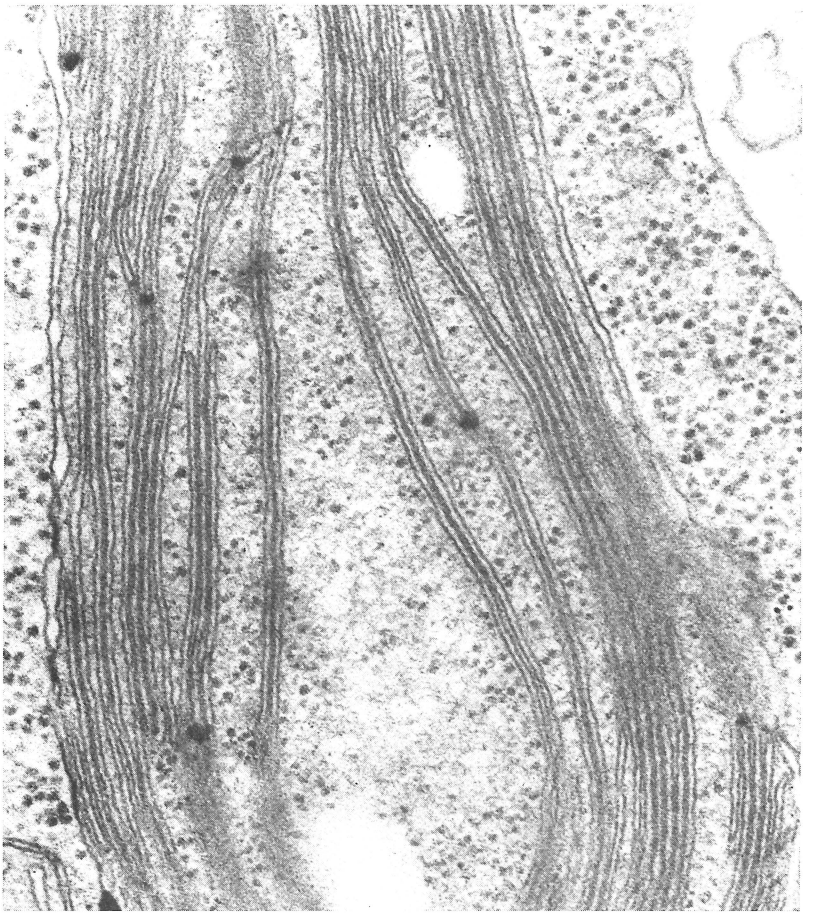


FIGURE 2. The fused and stacked chloroplast membranes (thylakoids) of wild-type *C. reinhardi*. Magnification: 113,900X.

either phototrophically or mixotrophically (Table 1).

When the fine structure of the chloroplasts of phototrophically-grown cells was examined (Figure 4), the chloroplast membranes were found to be fused and stacked (GOODENOUGH and STAEHELIN 1971), a result that would at first appear to support the hypothesis that the stacking of

chloroplast membranes is required for photosynthesis. The fact that the mutation in *ac-5* is conditional with regard to the fusion of chloroplast membranes allowed a direct test of the hypothesis. GOODENOUGH and STAEHELIN therefore tested the *in vivo* photosynthesis of *ac-5* cells having stacked or unstacked chloroplast membranes and found the rates to be identical at saturating light intensities (Table 1). They

Table I

Chlorophyll (chl) Content and Rates of Photosynthetic Reactions of the Wild-Type, *ac-5*, and *ac-31* Strains of *C. reinhardi.**

Data are taken from single, representative experiments.

Strain	Growth condition	Total chloro-phyll	Chloro-phyll a:b	CO_2 fixation		DPIP photoreduction		NADP photoreduction		NADP photoreduction from DPIP ascorbate	
		µg chloro-phyll per 10^6 cells		µmoles CO_2 fixed/hr per mg chloro-phyll	µmoles CO_2 fixed/hr per 10^6 cells	µmoles photo-re-duced/ hr per mg chl	µmoles photo-re-duced/ hr per 10^6 cells	µmoles photo-re-duced/ hr per mg chl	µmoles photo-re-duced/ hr per 10^6 cells	µmoles photo-re-duced/ hr per mg chl	µmoles photo-re-duced/ hr per 10^6 cells
Wild type	Mixo-trophic	3.6	2.5	152	0.551	201	0.725	101	0.367	90	0.325
	Photo-trophic	1.8	2.6	152	0.279	142	0.240	102	0.172	58	0.099
ac-5	Mixo-trophic	0.66	3.4	230	0.151	335	0.265	134	0.106	108	0.085
	Photo-trophic	0.61	3.0	230	0.140	356	0.196	128	0.070	120	0.066
ac-31	Mixo-trophic	1.1	3.4	184	0.202	265	0.169	232	0.148	91	0.058

*From U.W. Goodenough and L.A. Staehelin, "Structural differentiation of stacked and unstacked chloroplast membranes," J. Cell Biol., 1971. 48:594-619 (with permission).

also showed this to be true for certain *in vitro* partial photochemical reactions associated with either photochemical systems I or II (Table 1). Thus fusion and stacking of chloroplast membranes is not required for photosynthetic activity in *C. reinhardi*. There remains the possibility that the fusion and stacking of the membranes is somehow related to photosynthetic efficiency (GOODENOUGH *et al.* 1969), but this possibility remains to be tested rigorously.

THE INTERNAL STRUCTURE OF FUSED AND UNFUSED MEMBRANES

At the time that the conditional nature of the *ac-5* mutation was being explored, ARNTZEN *et al.* (1970) published a study of the internal structure of spinach chloroplast membranes as revealed by freeze-etch electron microscopy. It was known from studies of PARK and BRANTON (1966) that chloroplast membranes possess tightly packed arrays of unusually large particles, and ARNTZEN *et al.* demonstrated that when

spinach chloroplasts were treated with digitonin to yield several membrane fractions (ANDERSON and BOARDMAN 1966), a fraction rich in photochemical system II activity was also the exclusive location of the large particles. This observation suggested that the large particles corresponded to photochemical system II. Since the photochemical system II-rich fraction was also rich in stacked membranes, however, it was

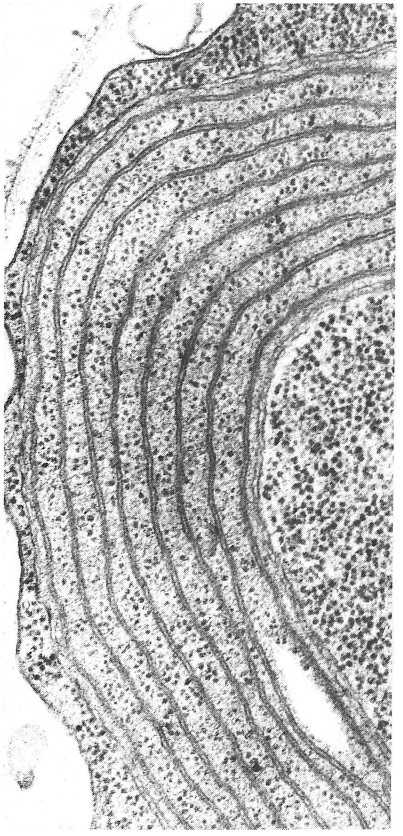


FIGURE 3. The unfused chloroplast membranes of mixotrophically-grown cells of *ac-5*. Magnification: 81,900X.

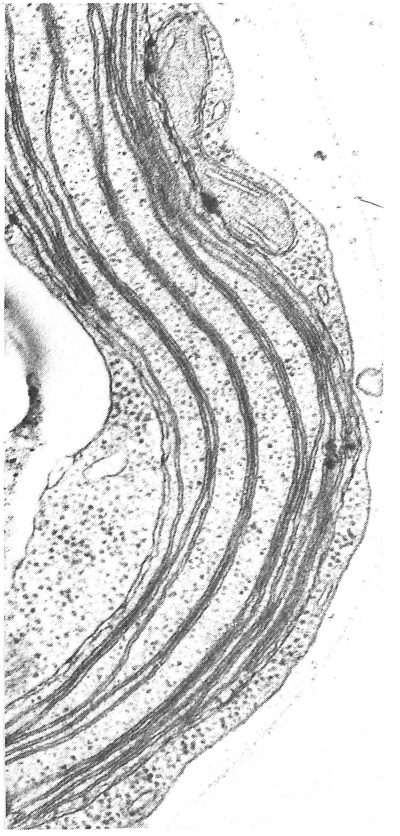


FIGURE 4. The fused chloroplast membranes of phototrophically-grown cells of *ac-5*. Magnification: 81,900X.

possible that the large particles instead represented a structural conformation assumed during membrane fusion. A test of these alternatives was possible by studying the freeze-etch structure of *ac-5* membranes, since it was known that the

membranes possessed the full photochemical system II activity whether stacked or unstacked. The results of this study (GOODENOUGH and STAEHELIN 1971) revealed that the large particles were in fact absent from unstacked membranes and present in stacked membranes.

At this juncture, therefore, it appeared that the mixotrophic *ac-5* chloroplast membranes suffered from some alteration in their membrane structure which disallowed membrane fusion and stacking and large particle formation without altering the functional properties of the membranes in any qualitative way. This alteration in structure, moreover, could be manipulated by a simple change in growth conditions. Thus the *ac-5* strain appeared ideally suited to a study of the structural organization of a biological membrane.

CHLOROPLAST MEMBRANE PROTEINS

The approach taken so far has been to compare the chloroplast membrane polypeptides of wild-type cells and *ac-5* cells grown phototrophically and mixotrophically. Among the chloroplast membrane proteins of *C. reinhardi*, several that function in the photosynthetic electron transport chain have been isolated and purified from acetone powders of chloroplast membranes (GORMAN and LEVINE 1966a and b). None of these appears to be affected in cells of *ac-5*. In addition, HOOBER (1970) has developed a procedure for extracting polypeptides from chloroplast membranes of *C. reinhardi* with SDS (Sodium dodecyl sulfate) and urea and analyzing the polypeptides by SDS polyacrylamide gel electrophoresis. We have used HOOBER'S procedures in our studies of *ac-5*.

Figure 5 shows the similarity between the profiles of polypeptides from chloroplast membranes of mixotrophically- and phototrophically-grown cells of the wild-type strain; in both cases the membranes are fused and stacked. The polypeptides labeled a, b, and c are of particular interest, for they have been shown (LEVINE *et al.* 1972) to be associated with a membrane fraction that is rich in photochemical system II activity. These polypeptides are affected in *ac-5* depending on growth conditions. Figure 6A shows a typical gel scan of chloroplast membrane polypeptides obtained from mixotrophically-grown cells of *ac-5* having unfused and unstacked membranes. There is a marked deficiency for polypeptides a, b, and c. On the other hand, these polypeptides are readily apparent among the preparations made from phototrophically-grown cells of *ac-5* in which the membranes are fused (Figure 6B).

Following these observations we carried out a series of experiments in order to determine whether the polypeptides were present in some cell fraction other than chloroplast membranes, for HOOBER (1972) has shown that these polypeptides are found among the soluble cell proteins when translation on chloroplast ribosomes is inhibited by chloramphenicol. We have examined by electrophoresis the polypeptides obtained

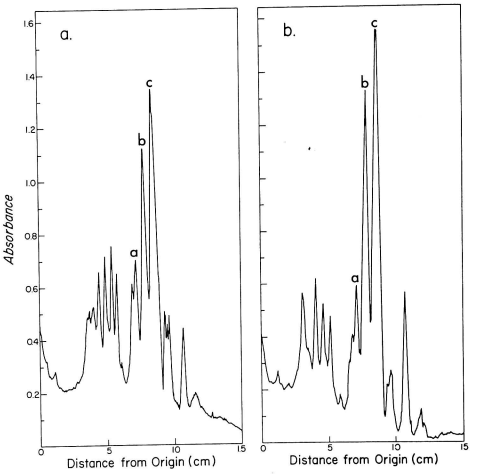


FIGURE 5. Electrophoretic separation of chloroplast membrane polypeptides from wild-type *C. reinhardi*. a. Mixotrophically-grown cells. b. Phototrophically-grown cells. Between 75 and 100 µg of protein were subjected to electrophoresis on SDS-polyacrylamide gels as described by HOOBER (1970) except that power was applied by a pulsed power supply operating at 140V and 20mA and 105 pulses/s. The gels were stained with Coomassie blue and scanned at 555 nm.

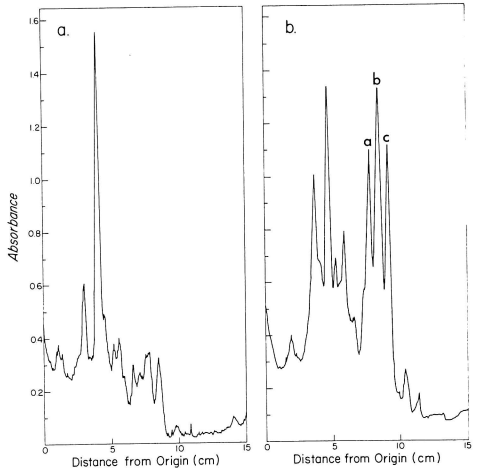


FIGURE 6. Electrophoretic separation of chloroplast membrane polypeptides from the *ac-5* mutant strain of *C. reinhardi*. a. Mixotrophically-grown cells. b. Phototrophically-grown cells.

from total cell protein, total membrane protein, and soluble protein from mixotrophically-grown cells of *ac-5* and polypeptides a, b, and c did not appear in any of the fractions.

Since the surfaces of the unfused and unstacked membranes of *ac-5* are more exposed than those of the fused membranes, we considered it possible that some or all of the polypeptides in question might be solubilized and lost during the preparation of the chloroplast membranes for electrophoresis. To test for this possibility, chloroplast membranes isolated from mixotrophically-grown cells of wild-type and phototrophically-grown cells of *ac-5* were unstacked by washing them five times in 0.05 M tricine NaOH, pH 7.3 (IZAWA and GOOD 1966). An aliquot of each preparation was examined by electron microscopy, and it was seen that the membranes had indeed become unstacked. The remaining material was used for electrophoresis. There was no significant loss of polypeptides a, b, or c from either wild-type or phototrophic *ac-5* membranes.

The absence of the three polypeptides from cells with unfused membranes might also be accounted for by the exposure of membrane surfaces to proteolytic enzymes during the isolation procedure. However, when fused and stacked wild-type membranes and unfused *ac-5* membranes were incubated with bovine serum albumin and casein (KUNITZ 1947) for various periods up to 19 hours at room temperature, there was no significant digestion of the proteins.

CONCLUDING REMARKS

The studies of chloroplast membranes of *ac-5* permit us to make several statements regarding the fusion of the membranes. It is clear from the work of GOODENOUGH and STAEHELIN (1971) that fused membranes contain arrays of large particles that can be visualized by freeze-etch microscopy and that unfused membranes of *ac-5* do not contain these particles. The unfused membranes of the mutant strain also lack at least three principal chloroplast membrane polypeptides. When the membranes of the mutant strain become fused in cells growing phototrophically, the particles and the polypeptides are both present. Thus there is a correlation between the fusion of the membranes, the presence of large particles, and the presence of certain polypeptides. The relationship between these features of membrane structure is as yet unknown, but it seems reasonable to adopt as a working hypothesis the concept that the polypeptides must be present in the chloroplast membrane if fusion is to occur and if large particles are to form as a consequence of the fusion process.

The genetic picture is more complex, for it appears that a single gene mutation affects the synthesis of several polypeptide chains. Three polypeptides appear to be affected in our studies, but HOOBER (personal communication) has recently shown by more sensitive procedures that additional polypeptides are encompassed by the peaks shown in the tracings of wild-type chloroplast membrane polypeptides (Figure 5).

Several interpretations of such a genetic picture are possible. The most obvious interpretation is that a deletion in *ac-5* eliminates a group of genes responsible for the synthesis of the membrane polypeptides; this possibility is most unlikely, however, since the mutant strain can synthesize the polypeptides under phototrophic growth conditions. Second, the genes responsible for the polypeptides might be under either positive or negative control and there might be a mutation in a regulatory gene that exacts such control. A diploid strain made between wild type and *ac-5* would show the mutant gene to be dominant if the control were negative and if the mutation were in a regulatory gene of the i^s type. There is at least one precedent for an i^s-like mutation in the eukaryotes, namely, the testicular feminizing mutation in the mouse (OHNO 1971). Since diploids of *C. reinhardi* can be made (EBERSOLD 1967), the possibility of a mutation in a gene exerting a negative control can be tested.

A third possibility is that the *ac-5* mutation could be in a gene which specifies a protein that is essential for the incorporation of polypeptides a, b, and c into chloroplast membranes during membrane assembly. A fourth possibility, that the *ac-5* protein is concerned with the transport of the polypeptides across the chloroplast envelope, seems unlikely since a number of other polypeptides synthesized in the cytoplasm are present in the chloroplast in normal amounts (ARMSTRONG *et al.* 1971). Since we did not find the polypeptides among those obtained from the soluble proteins, this possibility would seem to be excluded. We assumed, however, that the polypeptides in question would be continually synthesized and that they would be retained in cells in the logarithmic phase of growth, and this assumption may be incorrect. Should the mutation prevent the polypeptides from being incorporated into their normal sites, the polypeptides may ultimately be destroyed. With this idea in mind we have crossed *ac-5* with *y-1*, a mutant strain that cannot synthesize chloroplast membranes in the dark (SAGER and PLADE 1957). Our aim is to grow cells of the doubly mutant strain in the dark and then to place them in the light for ten hours during which time they will synthesize an entirely new complement of chloroplast membrane proteins (HOOBER 1970). We will then examine the soluble protein fraction for the polypeptides a, b, and c. If they are found, then we can rule out a regulatory gene mutation and can instead conclude that the *ac-5* mutation may play some direct role in chloroplast membrane assembly, a role that is in some obscure way affected by phototrophic *versus* mixotrophic growth conditions.

ACKNOWLEDGMENTS

The author wishes to acknowledge the advice and suggestions made by Professor Ursula Goodenough and the expert technical assistance of Ms. Heather Duram. The research described here is supported by grants from the National Science Foundation (GB 18666 and GB 29203).

LITERATURE CITED

ANDERSON, J.M. and N.K. BOARDMAN 1966 Fractionation of the photochemical systems of photosynthesis. I. Chlorophyll contents and photochemical activities of particles isolated from spinach chloroplasts. Biochem. Biophys. Acta 112:403-421.

ARMSTRONG, J.J., S.J. SURZYCKI, B. MOLL, and R.P. LEVINE 1971 Genetic transcription and translation specifying chloroplast components of Chlamydomonas reinhardi. Biochemistry 10:692-701

ARNTZEN, C.J., R.A. DILLEY, and F.L. CRANE 1969 A comparison of chloroplast membrane surfaces visualized by freeze-etch and negative staining techniques and ultrastructural characterization of membrane fractions obtained from digitonin-treated spinach chloroplasts. J. Cell Biol. 43:16-31.

EBERSOLD, W.T. 1967 Chlamydomonas reinhardi: Heterozygous diploid strains. Science 157:447-449.

GOODENOUGH, U.W. and R.P. LEVINE 1969 Chloroplast ultrastructure in mutant strains of Chlamydomonas reinhardi lacking components of the photosynthetic apparatus. Plant Physiol. 44:990-1000.

GOODENOUGH, U.W. and R.P. LEVINE 1970 Chloroplast structure and function in ac-20, a mutant strain of Chlamydomonas reinhardi. III. Chloroplast ribosomes and membrane organization. J. Cell Biol. 44:547-562.

GOODENOUGH, R.W. and L.A. STAEHELIN 1971 Structural differentiation of stacked and unstacked chloroplast membranes. J. Cell Biol. 48:594-619.

GOODENOUGH, U.W., J.J. ARMSTRONG, and R.P. LEVINE 1969 Photosynthetic properties of ac-31, a mutant strain of Chlamydomonas reinhardi devoid of chloroplast membrane stacking. Plant Physiol. 44:1001-1012.

GORMAN, D.S. and R.P. LEVINE 1966a Photosynthetic electron transport chain of Chlamydomonas reinhardi. IV. Purification and properties of plastocyanin. Plant Physiol. 41:1637-1642.

GORMAN, D.S. and R.P. LEVINE 1966a Photosynthetic electron transport chain of Chlamydomonas reinhardi. V. Purification and properties of cytochrome 553 and ferredoxin. Plant Physiol. 41:1643-1647.

HOMANN, P.H. and G.H. SCHMID 1967 Photosynthetic reactions of chloroplasts with unusual structures. Plant Physiol. 42:1619-1632.

HOOBER, J.K. 1970 Sites of synthesis of chloroplast membrane polypeptides in Chlamydomonas reinhardi. J. Biol. Chem. 245:4327-4334.

HOOBER, J.K. 1972 A major polypeptide of chloroplast membranes of Chlamydomonas reinhardi: Evidence for synthesis in the cytoplasm as a soluble component. J. Cell. Biol. 52:84-96.

IZAWA, S. and N.E. GOOD 1966 Effect of salts and electron transport on the conformation of isolated chloroplasts. II. Electron microscopy. Plant Physiol. 41:544-552.

KUNITZ, M. 1947 Crystalline soybean trypsin inhibitor. II. General properties. J. Gen. Physiol. 30:291-310.

LEVINE, R.P. 1969 The analysis of photosynthesis using mutant strains of algae and higher plants. Ann. Rev. Plant Physiol. 20:523-540.

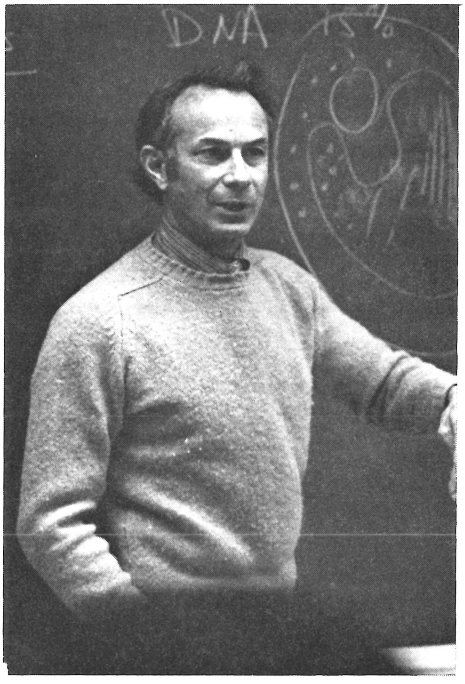
LEVINE, R.P., W. G. BURTON, and H.A. DURAM 1972 Membrane polypeptides associated with photochemical systems. Nature 237:176-177.

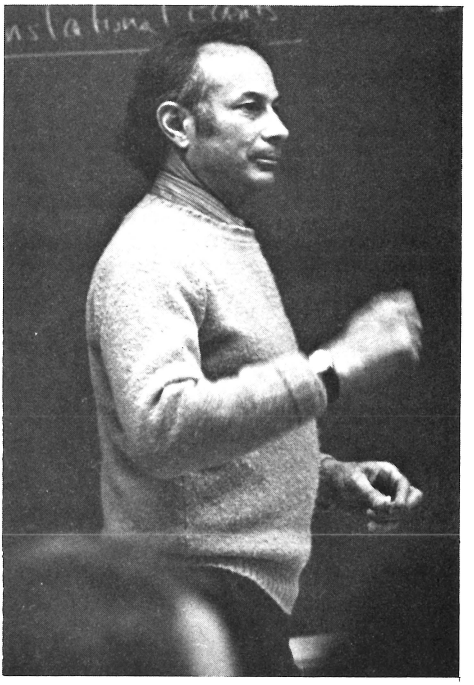
OHNO, SUSUMU 1971 Simplicity of mammalian regulatory systems inferred by single gene determination of sex phenotypes. Nature 234:134-137.

PARK, R.B. and D. BRANTON 1966 Freeze-etching of chloroplasts from gluteraldehyde-fixed leaves. Brookhaven Symp. Biol. 19:341-351.

SAGER, R. and G.E. PALADE 1954 Chloroplast structure in green and yellow strains of Chlamydomonas. Exptl. Cell Res. 7:584-588.

The audience





Dr. Levine at the Symposium