THE EFFECTS OF LECTINS ON CHLOROPLASTS ISOLATED FROM PISUM SATIVUM

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JULIA MAUREEN WRIGHT

THE EFFECTS OF LECTINS ON CHLOROPLASTS ISOLATED FROM *PISUM SATIVUM*

An Abstract

Presented to

the Graduate Council

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Julia Maureen Wright

May, 1983

ABSTRACT

To test the viability of using lectins in the study of organelle bi-layer outer membranes, chloroplasts were isolated from Pisum sativum and incubated with four lectins: Concanavalin A, Phaseolus vulgaris, Type V, Phytolacca americana Mitogen, and Pisum sativum, Type III. A variety of visual assessments and physiological methods were employed to test the effects of the lectins on chloroplast agglutination, mitogenesis, protein synthesis, and photosynthesis.

Each lectin increased agglutination of the isolated organelle. A lectin mitogenic effect was demonstrated in all but one incubation by a decrease in mean chloroplast size of from 10 to 30 percent. The effect of incubation with lectins on plastid protein synthesis levels varied with each lectin. Increases and decreases in protein synthesis were observed in incubations from each of the four lectins. Whether protein synthesis was enhanced or suppressed was lectin concentration dependent. Carbon incorporation from CO_2 was decreased, with the exception of Pisum sativum, Type III incubations in which it was greatly enhanced. No correlation was observed between the effect of lectins on carbon incorporation and on protein synthesis.

The results indicate that lectins can be used to manipulate organelle bi-layer outer membranes.

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To the Graduate Council:

I am submitting herewith a thesis written by Julia Maureen Wright entitled "Effects of Lectins on Chloroplasts Isolated from Pisum sativum." I recommend that it be accepted in partial fulfillment of the requirements for the degree Master of Science with a major in Biology.

D. L. Stone
Major Professor

We have read this thesis and recommend its acceptance:

Edward W. Hunter
Second Committee Member

Third Committee Member

Accepted for the
Graduate and Research Council:

William S. Ellis
Dean of the Graduate School

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CHAPTER I

INTRODUCTION

The outer membranes of certain organelles such as mitochondria and chloroplasts are of a bi-layer construction consisting of lipids, proteins, and sugars similar to that of plasma membranes (Avers, 1978).

Plasma membranes function in "intercellular communication, the regulation of cell growth, cell differentiation, and the immune response" (Sharon, 1977). These processes involve interactions of cell surface receptors, which utilize sugars of oligopolysaccharide chains of glycoproteins or glycolipids in the bi-layer (Sharon, 1974). These terminal sugars act as recognition sites on the cell surface. This recognition reaction is similar to the immune response.

One tool in studying the properties of plasma membranes has been the use of lectins (proteins derived mostly from plant materials) which bind specifically to the terminal sugar residues of carbohydrates bound to the cell surface (Sharon, 1977). Because of this specificity, testing with many different lectins yields information about the composition of specific sugar residues of a glycoprotein or glycolipid which functions as a binding site on the plasma membrane (Sharon, 1974).

It has long been known that lectins agglutinate erythrocytes (Sharon and Lis, 1972). More recent studies show that once the cell wall has been enzymatically digested away, lectins will also cause the agglutination of plant cells (Burgess and Linstead, 1976; Chin and Scott, 1979). Another physiologic phenomenon characteristic of

certain lectins is that of stimulating cell division when bound to the cell surface (Robbins, 1964).

There are numerous reports concerning the composition of plant plasma membranes (Mellor and Lord, 1979). Very little information has been reported concerning the physiological properties of glycoproteins from membranes in the interior of cells (Mellor and Lord, 1979).

The purpose of this study was to determine if lectins, which have proven valuable in the study of plasma membranes, could be of value in the study of the bi-layer outer membranes of cellular organelles. To test this possibility chloroplasts were isolated from Pisum sativum and incubated with four different lectins. The effects of these lectins on the chloroplasts were then monitored by a variety of visual assessments and physiological tests.

CHAPTER II

REVIEW OF THE LITERATURE

There are several sources of information available on the bi-layer construction of plasma membranes and the outer membranes of mitochondria and chloroplasts. Sharon and Lis (1972) described the nature of bi-layer membranes and how lectins interact with them. Later, Sharon (1974) described in greater detail the components of bi-layer membranes. Sharon (1977) further summarized how lectins bind to surface receptors, oligopolysaccharides of membrane glycoproteins and glycolipids. Later research indicates that lipid-linked sugars may be intermediates in plant cell glycoprotein synthesis (Mellor and Lord, 1979).

The plasma membrane was originally thought to function only as a permeability barrier. More recent studies have shown that the outer membrane of chloroplasts are also involved in active transport of molecules into the plastid. Guy et al. (1978) found a specific transport system for glucose in the outer membrane of Pisum sativum chloroplasts. Rudiger (1978) concluded that there are outer membrane translocators in chloroplasts which are unspecifically permeable to all nucleotides. He further stated that these carriers are already present in the envelope membranes of etioplasts. McLaren and Barber (1977) disagreed with the concept that isolated chloroplasts are fully permeable to amino acids. This was based on tests which showed a 98 percent inhibition of leucine incorporation with the addition of isoleucine to the medium. They concluded that chloroplasts have a carrier system specific for leucine. However, they noted that the

incorporation of other amino acids may be on a non-specific basis.

It has long been known that many lectins will cause agglutination of human blood cells (Liener, 1976). Later testing revealed that some lectins have the ability to agglutinate white blood cells and still others agglutinate plant cells from which the cell wall has been enzymatically digested away (Chin and Scott, 1979). With the cell wall removed, lectins stimulated the division of tobacco mesophyll cells but division ceased in the presence of 2 percent sucrose (Nagata and Takebe, 1970). A study of the lectin from Pokeweed (Phytolacca americana) revealed that it causes optimum agglutination of both red and white blood cells only in media of a greater density than those in which other lectins are most effective (Borjeson et al., 1966). A study of agglutination of higher plant protoplasts by Concanavalin A (Con A) revealed that the binding of the lectin results in a clustering of the cell surface receptor sites (Burgess and Linstead, 1976). Williamson et al. (1976), working with Con A agglutination of plant protoplasts, observed this receptor site clustering. They concluded that the agglutinating ability of Con A is pH dependent. At a neutral pH the protein is a tetramer with four saccharide binding sites for glucose or mannose. At a lower pH the molecule becomes a dimer and the binding sites are lost (Williamson et al., 1976).

Chin and Scott (1979), working with the phytolectins Con A, Phytohemmaglutinin, and wheat germ agglutin concluded that lectins agglutinate protoplasts indiscriminately and that they also stimulate incorporation of leucine into DNA and RNA, thus stimulating protein synthesis.

Protoplast agglutination by Con A and peanut lectins was studied by Larkin (1978), who concluded that lectins do not differentiate between plant protoplasts.

Many lectins are known to have a mitogenic effect on cells. Nowell (1960) observed the mitogenic property of phytohemmaglutinin (PHA), the lectin of the red kidney bean, when lymphocytes were treated with PHA. This treatment caused an increase in the number of blast cells and dividing cells.

The agglutinative and mitogenic specifications of lectins are such that they are useful clinically. Childress et al. (1979) observed that some lectins bind to both normal and cancerous cells and specifically agglutinate cancerous cells. They suggested that this agglutination was dependent upon cell size. Large tumor cells were agglutinated but small tumor cells were not. Elves and Wilkinson (1963) observed that PHA distinguishes between normal and leukaemic leukocytes in agglutination. Phytohemmaglutinin has been used to stimulated mitosis for chromosomal analysis (Robbins, 1964). The two lectins from gorse (Ulex europeus), when combined, are useful clinically in the diagnosis of secretors and identification of subgroups of the A and AB blood groups (Osawa and Matsumoto, 1972).

Most phytolectins have been isolated from members of the family Leguminosae (Liener, 1976). In a study of the amino acid sequences of these proteins, Hankins et al. (1979) observed that the legume lectins are evolutionarily closely related. This similarity was also reported in a study comparing lectins from peas and lentils (Foriers et al., 1977). Both lectins are composed of two alpha and beta poly-

peptide chains (Entlicher et al. 1970; Trowbridge, 1974; Foriers et al. 1977). Of the first twenty-five amino acids of the beta chains, only two differences were found between the pea and lentil lectins (Foriers et al. 1977).

Concanavalin A, the lectin from Jack Bean, has been the most widely studied lectin. Agrawal and Goldstein (1972) discussed the molecular structure of Con A and its properties, including its use to distinguish between normal and tumor cells.

The red kidney bean lectin, has been widely studied (Elves and Wilkinson, 1963). It can agglutinate both red and white blood cells and has a mitogenic ability with lymphocytes (Nowell, 1960). There is a disagreement about its molecular structure and weight. Generally, it is agreed that PHA is composed of one glycoprotein which agglutinates leucocytes and at least one but probably several proteins which can agglutinate both erythrocytes and leucocytes (Weber et al.,1972). Kornfield et al. (1972) reviewed the literature on PHA and concluded that there is confusion about its molecular structure because the lectin has been isolated in three forms. He labeled these forms: PHA-P, a mixture of all component lectins displaying all the noted lectin properties; L-PHA, which only agglutinates leucocytes; and E-PHA which only agglutinates erythrocytes. Kornfield stated that it is this final lectin, E-PHA, which stimulates lumphocytes to divide. In disagreement, Goldbert et al. (1969) reported that the lymphocytic stimulating factor from kidney bean is not a lectin or even a protein.

The lectin of the garden pea (Pisum sativum) was similar to Con A in that it must first bind Mn^{2+} then Ca^{2+} before binding sites for

saccharides are formed (Liener, 1976). One study showed that when Ca is removed from pea lectin it loses its ability to precipitate the sugar mannose. Also, without Ca its overall agglutination ability is reduced by 75 percent (Paulova et al., 1971). As with many lectins, there is disagreement over its molecular structure. Generally, it is believed that pea lectin is not a glycoprotein. Entlicher et al. (1970) claim that it has a very low amount of carbohydrate, 0.3 percent of its molecular weight being glucose. Further, they reported that pea lectin is actually two closely related proteins of similar molecular weight. Both are said to be non-specific haemagglutinins. Trowbridge (1974) also stated that pea lectin contains little carbohydrate (5 percent). He reported the lectin is two closely related proteins, but these he claims are composed of small and large polypeptide chain subunits.

Lis and Sharon (1973) detailed the molecular structure and physical properties of all lectins isolated at that time. A more current review by Liener (1976) discussed the possible function of lectins in plants and the possible role of legume lectins in the symbiotic relationship between root nodules and bacteria. Liener stated that lectins could be the cause of the toxicity and growth retardation of cattle which have eaten raw beans.

Hall (1972) defined the terms used to describe various types of chloroplast isolations. He observed the greatest CO_2 fixation rates with intact chloroplasts that had been isolated with relatively little debris. By studying the extent of lamellar membrane composition, it was determined that chloroplasts isolated from mature cotyledons are similar to chloroplasts isolated from mature leaves (Mares et al., 1979).

Panigrahi and Biswal (1979) conducted a study on the aging of isolated chloroplasts and determined that the DNA content is less affected than the RNA or protein contents with age.

The various methods of chloroplast isolation were reviewed by Givan (1979), who concluded that many of the problems in comparing *in vitro* analysis to *in vivo* conditions are due to the broken chloroplast pieces. He suggested using sucrose or sorbitol as an osmoticum in the isolation buffer to prevent chloroplast bursting. Elias and Givan (1979) published a method for chloroplast isolation in which Bovine serum albumin is added to the isolation medium to prevent soluble enzymes from adhering to the chloroplast membrane. The method employs a sucrose density gradient and involves ultra-centrifuging twice before a relatively clean chloroplast pellet is obtained. The chloroplast isolation method developed by Mills and Joy (1980) includes sorbitol and BSA in the isolation media, but the density gradient is formed by Percoll. Only one short centrifugation is necessary. The chloroplasts are isolated in 5 to 10 minutes with a high viability and a low amount of cellular debris.

According to Buterfass (1973) plastid division is independent of cell division but is still controlled by the nucleus. He observed that an increase in ploidy corresponded with an increase in the number of chloroplasts. Boffey *et al.* (1979) reported that the greatest amount of chloroplast division did not occur in the region of the basal meristem. They further noted that chloroplast DNA synthesis increased in the area of greatest chloroplast division and they concluded that chloroplast division is not linked to cell division.

Rose et al. (1974) stated that in higher plants all chloroplasts have the ability to divide and synthesize DNA, which is distributed equally among the daughter chloroplasts. Lamppa and Bendich (1979) found in studying Pisum sativum that the proportion of chloroplast DNA to the total plant DNA increases as the plant matures from young shoots to fully green leaves. Hirai et al. (1979) determined that plastid r-RNA synthesis is not related to cytoplasmic, nuclear r-RNA synthesis.

Rameriez et al. (1967) were the first to describe light driven protein synthesis in intact isolated chloroplasts. In one experiment using light as the energy source, Mills and Wilson (1978) showed that methionine is manufactured in chloroplasts and this synthesis is regulated by lysine and threonine. Blair and Ellis (1973) stated that the 70-S ribosomes of chloroplasts account for up to 50 percent of all ribosomes in a plant cell. Their experimentation indicated that the main product of pea chloroplast protein synthesis is the major subunit of ribulose diphosphate carboxylase. This is Fraction I protein, the most abundant plant protein, which is found exclusively in chloroplasts. Bottemley et al. (1974) stressed that a balance between Mg^{2+} and K^{+} ions in the isolation medium is necessary for light driven protein synthesis to occur in insolated chloroplasts. They isolated nine proteins as products of chloroplast protein synthesis, and the most abundant was the large subunit of Fraction I protein. Ellis (1977) proposed a model for the cooperation between chloroplast and nuclear genomes in the synthesis of Fraction I protein. In Fraction I, the major subunit is produced in the chloroplast and the minor subunits are produced in the cytoplasm.

Then the minor subunits enter the chloroplast, via an unspecified "messenger pathway," where the molecule is joined and stored.

CHAPTER III

MATERIALS AND METHODS

Plant Material

Chloroplasts were isolated from garden pea plants (Pisum sativum) grown from seeds obtained from the Carolina Biological Supply Company, Burlington, North Carolina. The plants were grown in vermiculite in a growth chamber with a 12/12 hour light regime at a temperature of 19°C. The plants were watered with distilled water ab libitum.

Isolation of Chloroplasts

The chloroplasts were isolated from 9 to 12 day old seedlings according to the mechanical method of Mills and Joy (1980). The procedure involves lysing the cells then isolating the chloroplasts by centrifugation of the homogenate in a Percoll density gradient.

The leaves and green upper shoots of the seedlings were chopped into roughly 1 cm pieces. These were added to a graduated cylinder containing 75 ml of extraction medium until a final volume of 100 ml was reached.

The chloroplast extraction medium consisted of 330 mM Sorbitol, 50 mM Tricine-KOH (pH 7.9), 2 mM EDTA, 1 mM $MgCL_2$, and 0.1 percent (w/v) BSA (Mills and Joy, 1980).

The plant material was then homogenized for 5 seconds with a Brinkmann Instruments Polytron homogenizer at a speed setting of five. The resultant brei was then filtered through two layers of Miracloth and two layers of cheese cloth. Thirty milliliters of the cleared homogenate were then poured into each of the two 50 ml centrifuge tubes and underlayered with 14 ml of a Percoll medium consisting of 40

percent (v/v) Percoll, 330 mM Sorbitol, 50 mM Tricine (pH 7.9), and 0.1 percent BSA. The chloroplasts were then isolated by centrifugation at 2,500 x g for one minute in an International Refrigerated Centrifuge model B-20 with an 850 rotor head. Following isolation each resultant chloroplast pellet was resuspended in 12 ml of a sustaining medium for experimental procedures. This chloroplast suspension medium was formulated by Anderson and Done (1977) and consisted of 0.33 M Sorbitol, 2mM Na_2EDTA, 1 mM $MgCl_2$, 50 mM HEPES, and 0.1 percent (w/v) BSA (pH adjusted to 7.9 with KCl).

Incubation of Chloroplasts with Lectins

Prior to each diagnostic procedure, isolated chloroplasts were incubated with each of the lectins. Four lectins (Concanavalin A, Phaseolus vulgaris, Type V, Phytolacca americana Mitogen, and Pisum sativum, Type III) were used for the subsequent chloroplasts studies. These lectins were purchased from Sigma Chemical Co., St. Louis, Mo.

Concanavalin A is the lectin from Jack Bean. It was selected for study because it was the first lectin to be obtained in crystalline form. There is considerable literature describing its biological effects on cells (Sharon and Lis, 1972). Phaseolus vulgaris, Type V, Kidney Bean Lectin, and Phytolacca americana Mitogen, Pokeweed lectin, were used due to their agglutinative and mitogenic effects on cells (Sharon and Lis, 1972). Pisum sativum, Type III was selected because it is a lectin from the plant from which the chloroplasts were to be isolated.

Each lectin was dissolved in Tricine-KOH (pH 7.9) in the following concentrations: 0.01 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, and 250 µg/ml. When 1 ml of chloroplasts sus-

pension was combined with 1 ml of dissolved lectin, this gave final lectin working concentrations of: 0.005 µg/ml, 0.05 µg/ml, 0.5 µg/ml, 2.5 µg/ml, 5 µg/ml, 25 µg/ml, 37.5 µg/ml, 50 µg/ml, and 125 µg/ml. Controls were 1 ml chloroplasts suspension plus 1 ml Tricine-KOH.

Labeling of Protein Synthesis

The effect of each lectin on protein synthesis was determined by incubating isolated chloroplasts with ^{3}H-Leucine added to every concentration of each lectin dilution series.

The incorporation of the radioactive amino acid into protein was measured by a modified form of the method developed by Mans and Novelli (1961). One milliliter of chloroplast suspension was added to 1 ml of each lectin concentration in Pyrex test tubes. To each incubation mixture 0.1 ml of ^{3}H-Leucine (5 uci/ml) was added. The samples were placed in a 30^{o}C waterbath for 30 minutes. After this time, protein synthesis was stopped and the protein was percipitated by the addition of 4 ml of cold TCA.

The incubation mixture was then filtered through a Millipore filtration system, trapping the samples on glass fiber filter papers. In order to displace any unincorporated ^{3}H-Leucine, the samples were then rinsed with three 5 ml alliquots of 5 percent TCA containing cold Leucine at a concentration of 100 µg/ml.

As in the Mans and Novelli (1961) procedure, the samples were heated in 5 percent TCA to hydrolyze and extract RNA and amino acid charged S-RNA. But the incubation time and temperature were changed. Samples were covered with 5 percent TCA and heated in a 50^{o}C waterbath for 10 minutes. Following incubation, the protein was percipitated with fresh 5 percent TCA.

The samples were then subjected to two more incubations to remove TCA and lipid material. The filter papers were covered with 95 percent ethanol and the test tubes were placed in a 60° to 70°C water-bath for 3 minutes. The 95 percent ethanol was poured off and a 2:2:1 mixture of ethanol:ether:chloroform was added. The samples were then heated at 55° to 65°C for 3 minutes.

This incubation mixture was poured off and the filter papers were washed with acetone to insure removal of the solvents and alcohol which may contribute to quenching.

The filter papers were then placed under a heat lamp and allowed to dry thoroughly before being placed in glass scintillation vials and covered with a toluene based liquid scintillation cocktail. The tritium labeled samples were counted for 10 minutes in a Nuclear Chicago Unilux III Liquid Scintillation System.

Protein Determination

The protein content of the chloroplast suspensions was determined by the Standard Assay Procedure of the Bio-Rad Protein Assay developed by Bio-Rad Laboratories (1979). Sample protein concentration was determined using a standard curve established with BSA dissolved in the Anderson-Done chloroplast suspension medium.

Agglutination

The chloroplast agglutinating ability of each lectin was judged indirectly by counting the decrease in the number of single chloroplasts in the total population.

Following the procedure of Larkin (1978), 1 ml of chloroplast suspension was added to 1 ml of each lectin dilution in a plastic petri dish. The dishes were rocked at room temperature for 20 to 40

minutes on a Yankee Variable Speed Rotator, setting 2. Following incubation, agglutination was assessed by a subjective scale using a light microscope on 40X and a haemocytometer.

The scale chosen was that developed by Grimulus, et al. (1974) wherein agglutination is rated from 0 to 4 by scoring the size of the mass of agglutinated cells. In this research, a single chloroplast rated 0; 2 to 15 chloroplasts adhered one to another rated 1; 16 to 25 rated 2; 26 to 50 rated 3, and masses judged to contain more than 50 chloroplasts rated 4.

Mitogenic Effect

Measurements of chloroplast size were taken for use as indicators of the lectins influence on chloroplast division. In theory, an increase in smaller chloroplasts following incubation with a lectin indicated that division had been enhanced (Boffey et al., 1979).

Following the same procedure as when assessing agglutination, chloroplasts were incubated with lectins for 20 to 40 minutes. From 100-150 measurements were made of chloroplasts incubated with each lectin dilution using a light microscope and a scanning scale micrometer.

Chlorophyll Determination

To determine any changes in the amount of chlorophyll present following incubation with lectin, the chlorophyll content of the isolated chloroplasts was assessed immediately when incubated with the lectins, then assayed again after 30 minutes. Chlorophyll was assayed by the procedure and formula of Aron (1949) who relied upon earlier discoveries. To test each sample, 0.5 ml of the usual 2 ml chloroplast-lectin incubation mixture was added to 9.5 ml of

80 percent acetone. Chlorophyll was estimated by absorption read at 645 and 663 nm.

Determination of CO_2 Production

For an assessment of the effects of the lectins on photosynthesis, CO_2 fixation was monitored by adding $NaH^{14}CO_3$ to the reaction mixture. This test was run for selected lectin concentrations chosen because they enhanced or depressed protein synthesis.

The 2 ml chloroplast-lectin incubation mixture of each sample was added to a rubber-stoppered Pyrex test tube. Four controls, two for light and two for dark, plus an additional sample for chlorophyll determination, were prepared by adding 1 ml of buffer to 1 ml of isolated chloroplasts in Anderson-Done media. The test tubes of the dark controls were wrapped in aluminum foil. All test tubes were placed in a glass water tank at room temperature. To acclimate the samples to these conditions, the tank was covered and the lab darkened for three minutes.

Following the dark period, 1 ml of $NaH^{14}CO_3$ (25 uCi), (0.25 uCi/ml) was added to each sample and the test tubes were stoppered again. Instead of $NaH^{14}CO_3$ 1 ml of distilled water was added to the samples reserved for chlorophyll determination. The holding tank was then surrounded by 150 watt fluorescent lights for 15 minutes. The samples were agitated during incubation.

After incubation with the $NaH^{14}CO_3$, the samples were removed from the tank and placed in a safety hood where the stoppers were removed and 0.5 ml of 1N HCl was added to each sample. Thus the reaction was stopped and excess $NaH^{14}CO_3$ was released. The samples remained under the hood for 15 minutes and were agitated every few

minutes during this period. Finally, the samples were filtered through a Millipore filtration system and rinsed five times with 5 ml alliquots of distilled water. The rinsate was necessary to remove any unincorporated $^{14}CO_2$. The filter papers were dried under a heat lamp for a minimum of one hour before being placed in glass liquid scintillation vals and covered with a toluene based counting cocktail. Samples were counted for 10 minutes.

Chlorophyll determination was made following the directions of Cherry (1973). One ml of the sample was added to 9 ml acetone and the mixture centrifuged. The amount of chlorophyll was estimated by reading absorption at 645 and 663 nm then computing the chlorophyll content by the formula of Aron (1949).

Significant differences among means of experimental measurements were determined by Duncan's Multiple Range (Duncan, 1955).

CHAPTER IV

RESULTS

Microscopic Observations

Chloroplast size measurement. Observed chloroplasts ranged in size from 0.465 um to 4.186 um. According to Avers (1978), mature chloroplasts range in size from 1.0 μm to 10.1 μm and proplastids, which may be clear or pale green, have a size range of 0.5 μm by 1.0 μm to 1.0 μm by 1.5 μm. The mean plastid size in each lectin concentration is given in Table I.

Of all the properties studied, the effect of lectin incubation on chloroplasts population mean size was the most consistent among the four lectins. With the exception of Phaseolus vulgaris, Type V, at a concentration of 5 μg/ml, each lectin incubation resulted in a reduction in plastid mean size of from roughly 10 to 30 percent of control mean chloroplast size (Table I). It can also be seen in Table I that plastid mean size at higher lectin concentrations was greater than the mean size at lower concentrations for Con A and Phytolacca americana Mitogen. In Phaseolus vulgaris incubations, chloroplast mean size decreased with increased lectin concentration. These fluctuations in plastid mean size at greater lectin concentrations are due to changes in the number of small plastids present. Table II shows at lectin concentrations of 37.5 μg/ml through 125 μg/ml, there was an increase in the number of chloroplasts 0.93 μm or smaller in PHA incubations. At these same concentrations, there was a decrease in the number of smaller plastids in both Con A and PkM incubations. Only in Pisum sativum, Type III incubations did

Table I. Effects of lectins on mean chloroplast size.

Lectin	Mean Chloroplast Size (um)								
	Lectin Concentration (ug/ml)								
	0.005	0.05	0.5	2.5	5	25	37.5	50	125
*Con A	1.544	1.507	1.628	---	1.536	1.674	1.842	1.888	1.758
PHA	1.736	1.774	1.758	1.762	2.077	1.465	1.502	1.591	1.475
PkM	1.454	1.374	1.378	1.389	1.349	1.374	1.631	1.696	1.71
Ps	1.541	1.589	1.56	1.609	1.549	1.628	1.49	1.536	1.623

*Lectins: Con A (Concanavalin A), PHA (Phythohemmaglutinin, Phaseolus vulgaris), PkM (Pokeweed, Phytolacca americana Mitogen), Ps (Pisum sativum). Mean size of chloroplast in control medium was 1.97 um.

Table II. Effects of lectins on percent of chloroplast population that was 0.93 um or smaller.

Lectin	Lectin Concentration (ug/ml)								
	0.005	0.05	0.5	2.5	5	25	37.5	50	125
*Con A	27.0	30.0	15.0		28.0	15.0	4.0	6.0	4.0
PHA	14.67	11.33	6.67	8.33	17.33	16.0	30.0	27.0	34.0
PkM	16.67	24.67	28.67	25.33	28.67	28.0	10.56	10.0	8.54
Ps	31.25	30.21	16.0	24.0	28.0	29.17	26.0	26.04	26.04

*Lectins: Con A (Concanavalin A), PHA (Phytohemmaglutinin, Phaseolus vulgaris), PkM (Pokeweed, Phytolacca americana Mitogen), Ps (Pisum sativum), Of control chloroplasts, 10.87% 0.93 um or smaller.

Table III. Chloroplast agglutination scores assessed by subjective scoring method.

Lectin	Agglutination Scores Lectin concentration (ug/ml)									
	0	0.005	0.05	0.5	2.5	5	25	37.5	50	125
*Con A	1	1	1	2	---	2	3	1	1	1
PHA	1	1	1	2	1	2	2	1	1	1
PkM	1	1	1	1	1	1	1	1	1	2
Ps	1	1	1	1	1	1	1	1	1	1

*Lectins: Con A (Concanavalin A), PHA (Phytohemmaglutinin, Phaseolus vulgaris), PkM (Pokeweed, Phytolacca americana Mitogen), Ps (Pisum sativum).

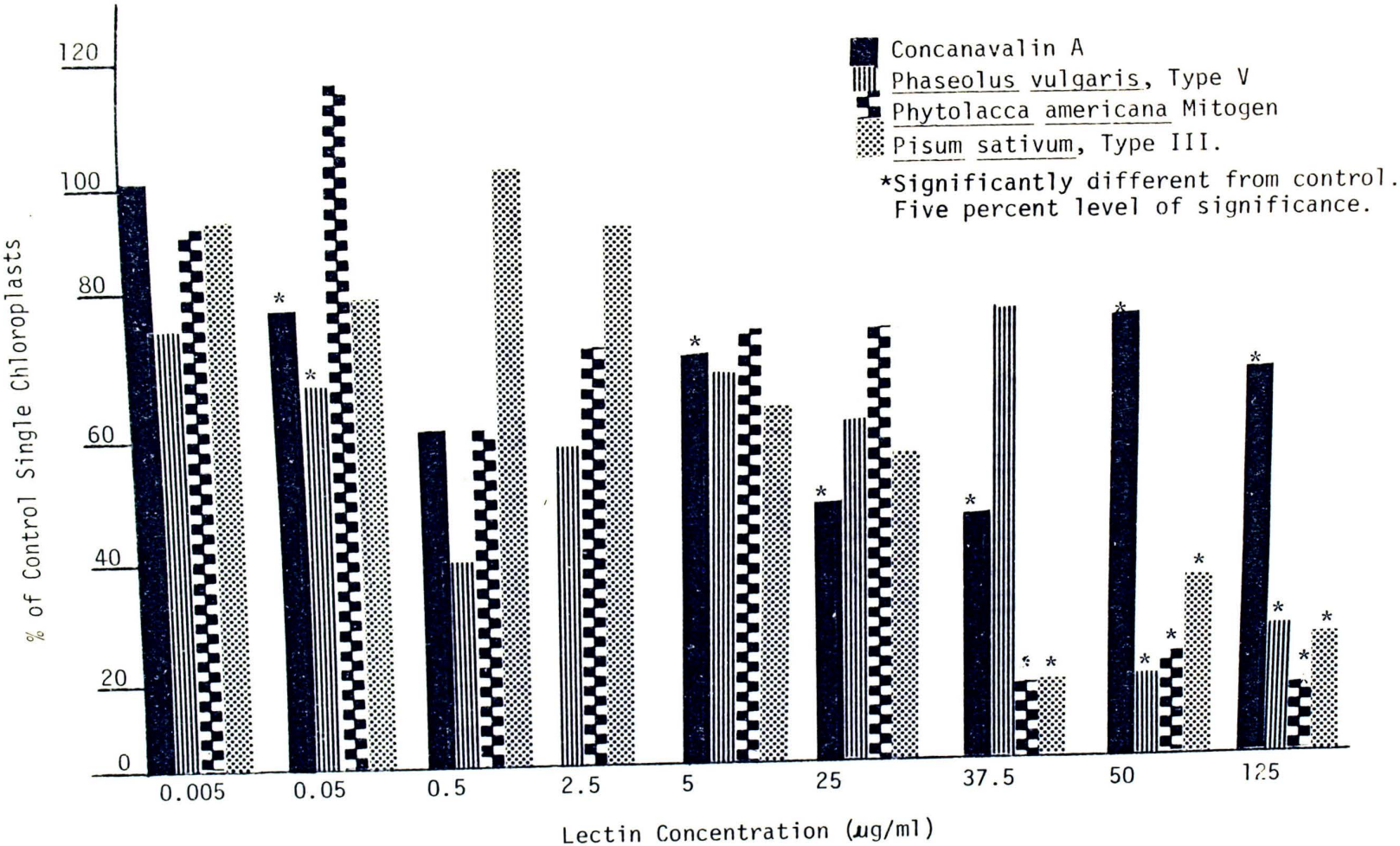

Figure 1. Effects of lectins on agglutination of chloroplasts. (Single chloroplasts expressed as a percentage of the number of single plastids in the control).

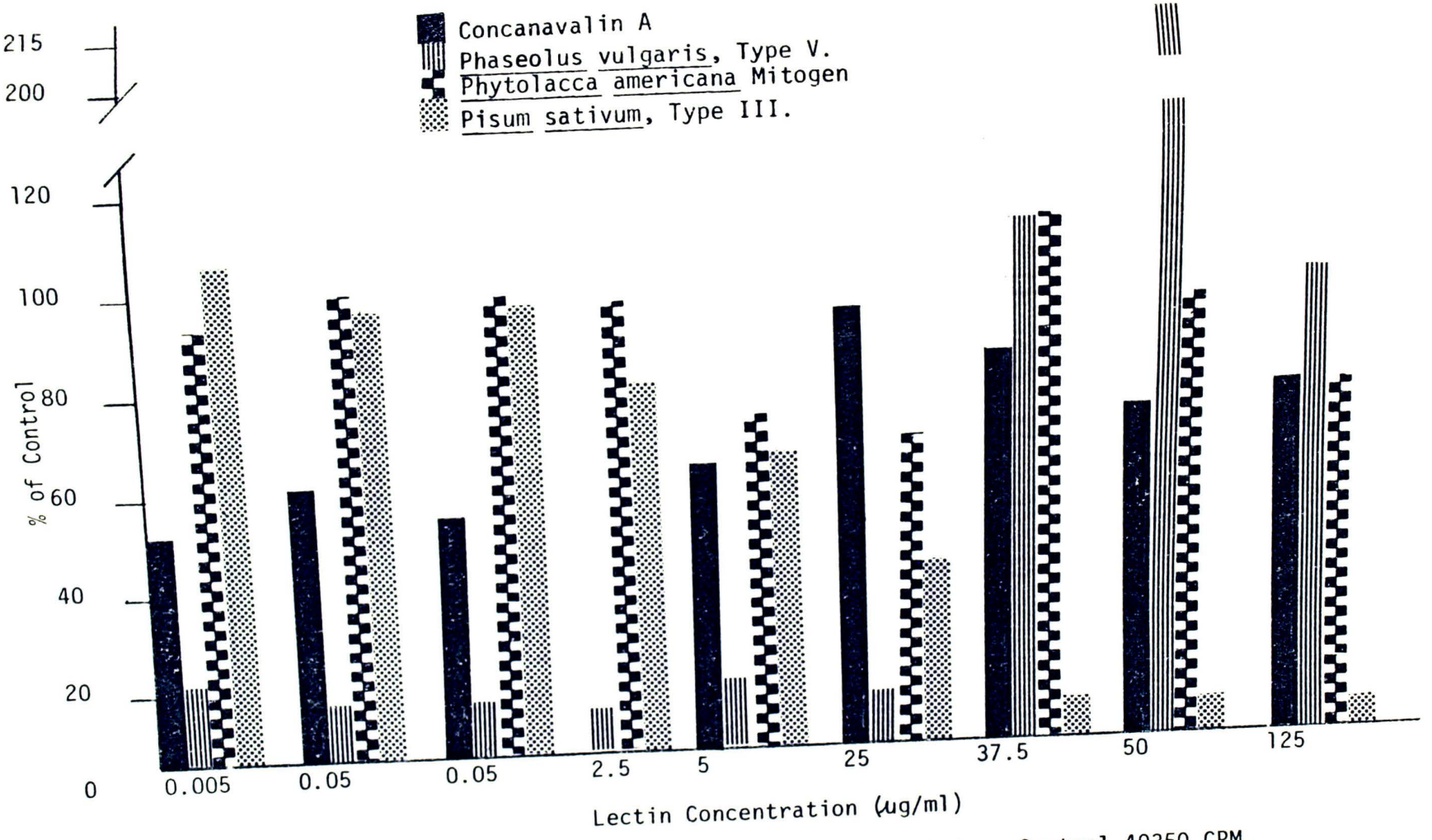

Figure 2. Effect of lectins on ^{3}H-Leucine incorporation into protein. Control 40350 CPM.

the percentage of smaller plastids remain fairly consistent, ranging from 24.0 to 31.25 percent (Table II).

Chloroplast agglutination. Agglutination was observed in every incubation including the control medium. Oppenheimer and Odencrantz (1972) also observed agglutination in the control medium. The percentage of single chloroplasts in the population was found to be a better indicator of agglutination that the subjective scoring method since random sampling produced an erratic pattern of scores (Table III). When expressed as a loss in the number of single chloroplasts, all lectins increased agglutination except for Phytolacca americana Mitogen, concentration 0.05 ug/ml (Figure 1). However, according to Duncan's Multiple Range satistical analysis, only the agglutination in the following lectin concentrations was significantly different from the control: Con A 0.5, 5, 25, 37.5, 50, and 125 µg/ml; PHA 0.5, 50, and 125 µg/ml; PkM 37.5, 50, and 125 µg/ml; and Ps 37.5, 50, and 125 µg/ml. As can be seen in Figure 1, there was a great increase in agglutination at higher lectin concentrations with PHA, PkM and Ps. With each lectin, there was a dramatic shift in the agglutination response between concentrations 25.0 µg/ml and 50 µg/ml.

Radioactive Labeling

^{3}H-Leucine incorporation - protein synthesis. Lectins were observed to increase protein sysnthesis at some concentrations while decreasing synthesis at others (Figure 2). The most erratic pattern of increasing and decreasing synthesis at different concentrations was that observed with PkM. With Con A, the lowest synthesis levels were observed in lower concentrations and near 100 percent control synthesis occurred at 25 µg/ml. In lower PHA concentrations, protein

Table IV. Effects of lectins on carbon fixation.

Lectin	Lectin concentration	cpm/ug chlorophyll	% of Control CPM	^{3}H-Leucine incorporation Greater than, + Less than, - Control
Concanavalin A	25.0	5.553	54.242	=
	50.0	7.25	70.823	-
Phaseolus vulgaris, Type V.	0.5	7.237	70.694	-
	2.5	6.211	60.668	-
	5.0	7.842	76.607	-
Phytolacca americana Mitogen	0.05	5.053	49.357	+
	5.0	2.605	25.45	-
	50.0	4.789	46.787	+
	125.0	5.75	56.17	-
Pisum sativum, Type III.	0.5	6.75	65.938	+
	5.0	25.461	248.715	-

synthesis was repressed, whereas in concentrations 37.5 µg/ml or higher synthesis was stimulated. The greatest synthesis observed, 214.36 percent of control, occurred in 50 µg/ml of PHA. The least synthesis, 0.98 percent of control, was observed in 125 µg/ml of Ps. Protein synthesis was enhanced at the lowest Ps concentrations but was severely repressed at concentrations greater than 37.5 µg/ml.

Carbon incorporation - photosynthesis. For this test, lectin concentrations in which protein synthesis had been enhanced or inhibited were chosen. This procedure was selected in an attempt to assess whether a correlation existed between the effect of lectins on protein synthesis and carbon incorporation. The greatest carbon incorporation per microgram of chlorophyll was observed in Ps incubations, 5 µg/ml and 25 µg/ml. These were the only samples in which incorporation was enhanced. These were concentrations at which protein synthesis was inhibited. However, no true correlation between carbon incorporation and protein synthesis levels was observed (Table IV). In all other samples, regardless of whether this lectin concentration yielded an increase or decrease in protein synthesis, the ^{14}C incorporation was less than that of the control.

CHAPTER V

DISCUSSION

Few general trends were noted concerning the physiologic responses of chloroplasts to incubation with lectins. Concanavalin A, Phytolacca americana Mitogen, and Pisum sativum, Type III had similar effects on mean plastid size, agglutination, and protein synthesis at a concentration of 5 µg/ml. With each of these lectins at this concentration, the average plastid size was 65 to 80 percent of control mean size, agglutination was near 40 percent greater than control, and protein synthesis was 67 to 84 percent that of control. Phytohemmaglutinin had a 38 percent increase in agglutination at this concentration but the observed protein synthesis and mean plastid size values were widely different than those observed with other lectins.

Chloroplast size measurement

Incubations with each of the four lectins resulted in a decrease in mean plastid size, due to an increase in the number of smaller plastids observed in higher concentrations of lectins, Con A, PHA, and PkM. This indicates a mitogenic effect on the chloroplasts by these lectins. Boffey (1979), when studying chloroplast division in wheat (Triticum aestivum) leaves, observed a 200 percent increase in the number of chloroplasts in leaf regions of active plastid division. He also noted that average chloroplast length in the most active division regions was 3.2 µm, compared to average chloroplast length of 4.7 µm to 5.2 um in nondivision regions.

In this study, both chloroplast size and the percentage of chloroplasts 0.93 micrometers or smaller indicate that the greatest Con A mitogenic influence occurs at a lectin concentration of 0.05 µg/ml. This is in disagreement with an observance of a maximum Con A mitogenic effect at concentration 5 µg/ml (Lis and Sharon, 1973). Several studies have indicated that Con A mitogenic ability is lost in acidic conditions and at temperatures lower than 25°C (Liener, 1976). However, these conditions did not factor in the results since this experimentation was conducted at room temperature and at p^H 7.9.

Lis and Sharon (1973) state that PHA has its greatest mitogenic effect on leukocytes in a concentration range of 1 to 10 µg/ml. In this concentration range of PHA, the least division of chloroplasts was observed.

Lis and Sharon (1973) did not consider Pisum sativum lectin to enhance mitogensis. In disagreement, Trowbridge (1974) lists this lectin as having mitogenic capabilities. Paulova et al. (1971) also report mitogenic activities by Ps lectin and state that it is inhibited by EDTA. In this experimentation, mitogenic capability was indicated but was not related to Ps concentration. In both the extraction and chloroplast suspension mediums, 2 mM EDTA was included.

Borjeson et al. (1966) observed a greatest PkM mitogenic effect at a concentration of 0.05 µg/ml. In this experimentation, the smallest mean size and most chloroplasts 0.93 micrometers or smaller were found in a PkM concentration range of 0.05 µg/ml to 25 µg/ml (Table I and Table II).

Chloroplast agglutination

Agglutination was clearly demonstrated. This means that either glycoprotein, glycolipid, or both surfaced receptors for each of the test lectins are present on the chloroplast outer membrane. Since Con A and Ps have been demonstrated to bind glucose, frucose, and mannose (Liener, 1976) and PHA binds galactose (Larkin, 1978), these sugars must be present as a part of the surface receptors. However, no further statement about the outer membrane receptor composition can be made without additional study. The experiments in this study do not indicate the relative abundance of any receptor. Also, it cannot even be concluded that these are terminal sugars of the oligopolysaccharide side chains since it is now known that lectins need not bind to a terminal sugar (Hankins et al., 1979; Paulova et al., 1971).

Greatest agglutination of chloroplasts by Con A, as determined by percentage of control single chloroplasts (Figure 1), was observed at a concentration of 37.5 µg/ml. The greatest agglutination by Con A when analyzed by the subjective scoring method (Table IV) occurred in the range of 0.5 µg/ml to 25 µg/ml. Chin and Scott (1979) observed greatest agglutination of wheat (Triticum aestivum) protoplasts by Con A at a lectin concentration of 100 µg/ml. Oppenheimer and Odencrantze (1972) state that the lowest Con A concentration at which maximum agglutination of plant protoplasts will occur is 50 µg/ml. In this survey of lectins known to have some agglutinative properties, Oppenheimer and Odencrantze (1972) observed that not all these lectins were able to agglutinate plant protoplasts. Sharon and Lis (1973) state that Con A at a concentration of 25 µg/ml will agglutinate malignant but not normal cells.

The greatest chloroplast agglutination by PHA occurred in 50 µg/ml of the lectin. Phytohemmaglutinin agglutination of leukocytes has been observed since the early 1900's (Kornfield et al., 1972).

The fewest single chloroplasts with Ps were observed in Ps concentrations greater than 37.5 µg/ml. Entlicher (1970) observed the most agglutination of red blood cells in a Ps concentration of 2.5 µg/ml. Paulova et al. (1971) state that 25 percent of agglutination by Ps is lost with the addition of EDTA to the medium.

Borjeson et al. (1966) also noted a decrease in agglutination with the addition of 0.003 M EDTA to the medium when observing lymphocyte agglutination by PkM. He further states that while the greatest agglutination with most lectins occurs in the presence of high molecular weight substances, the greatest agglutination by PkM occurs in saline. In this study the least agglutination of chloroplasts by PkM was observed in a PkM concentration of 0.05 µg/ml. Agglutination greatly increased in PkM concentration 37.5 µg/ml or more.

^{3}H-Leucine incorporation - protein synthesis

With the incorporation of ^{14}C-Leucine by barley (Hordeum vulgare) protoplasts incubated in Con A and PHA, Chin and Scott (1979) observed maximum protein synthesis at a concentration of 100 µg/ml for both lectins. The concentrations at which maximum ^{3}H-Leucine incorporation was observed with each lectin in this experimentation were: Con A 25 µg/ml, PHA 50 µg/ml, PkM 50 µg/ml, and Ps 0.005 µg/ml. While Con A, PHA, PkM, and Ps, all affected protein synthesis, how the lectins contributed to the observed fluctuations in synthesis was not determined.

The main product of pea chloroplast protein synthesis is the major

subunit of ribulose diphosphate carboxylase (Fraction I Protein) (Blair and Ellis, 1973). The smaller subunits of this protein are produced on ribosomes in the cytoplasm (Ellis, 1977). These smaller subunits enter the chloroplast and are joined to the major subunit to complete the protein (Ellis, 1977). Ellis states that some of these smaller subunits may direct the synthesis of the larger subunit. If this is so, lectins may affect chloroplast protein synthesis by blocking or enhancing the passage of Fraction I protein small subunits into the chloroplast.

Another possibility is that lectins inhibit or increase chloroplast protein synthesis by changing the permeability of the outer membrane to amino acids. McLaren and Barber (1977) state that leucine enters chloroplasts via a carrier system. If this is the case, the lectins could be competing for or blocking the carrier molecule to decrease synthesis. It must be noted that Rudiger (1978) found no such carrier system in his work and concluded that the chloroplast outer membrane is unspecifically permeable to nucleotides. If this is correct, it is still possible that lectins block or enhance the permeability of the membrane to leucine. It has been shown that agglutination of cells with lectins results in a clustering of the cell surface receptor sites (Burgess and Linstead, 1976). In this experimentation, agglutination increased while protein synthesis dropped significantly at lectin concentrations 37.5 µg/ml through 125 µg/ml with Pisum sativum, Type III. However, increased agglutination with decreased protein synthesis was not observed with the other lectins.

Carbon incorporation - photosynthesis

In each of the selected concentrations of Con A, PHA, and PkM,

carbon incorporation by the plastids was inhibited. In two Ps concentrations, 5 µg/ml and 25 µg/ml, carbon incorporation was greatly enhanced. No correlation was observed between ^{3}H-Leucine incorporation and carbon incorporation. Much more experimentation is needed before further statements can be made concerning the influence of lectins on CO_2 fixation by isolated chloroplasts.

CHAPTER VI

SUMMARY

Chloroplasts isolated from Pisum sativum were incubated with four lectins: Concanavalin A, Phaseolus vulgaris, Type V, Phytolacca americana Mitogen, and Pisum sativum, Type III. Visual assessment was made of lectin effect on plastid division and the agglutination of chloroplasts by lectins. Protein synthesis and carbon incorporation by isolated chloroplasts incubated with the lectins was also monitored by incorporation of ^{3}H-Leucine and carbon-^{14} dioxide respectively.

In all but one lectin incubation, the mean plastid size was 10 to 30 percent smaller than that of chloroplasts in the control medium. The exception was PHA concentration 5 µg/ml in which the mean size was slightly (5.5 percent) greater than that of the control. The least mean plastid size (68.4 percent of control) was observed in PkM concentration 5 µg/ml.

Increased agglutination was observed in every lectin incubation except PkM concentration 0.05 µg/ml, in which agglutination approximated that of control. The greatest agglutination (90 percent fewer single chloroplasts than control) was observed in PkM concentration 125 µg/ml.

With lectin incubation, plastid protein synthesis increased at some concentrations and decreased at others. The greatest synthesis, more than twice that of control, was observed in PHA concentration 125 µg/ml. Protein synthesis was inhibited the most, to less than 1 percent of control, in Ps concentration 125 µg/ml. In Ps incubations the greatest agglutination and the least protein synthesis were observed

in concentrations 37.5 µg/ml and greater. However, increased agglutination in conjunction with decreased protein synthesis was not observed with the other lectins.

Carbon incorporation was enhanced in only two of the selected lectin concentrations, Ps 5 µg/ml and 25 µg/ml. In these incubations, carbon incorporation was 249 percent and 322 percent of control respectively. No correlation was observed between protein synthesis levels and fluctuations in carbon incorporation.

Since all four lectins affect the physiology of the isolated chloroplasts, it is concluded that receptor sites for these lectins are present on the plastid outer double membrane surface. Though the lectins bind specifically to certain sugars, no further statement about the membrane compositon can be made without further research.

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