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# A Test of the Binary Chloroplast Membrane Hypothesis by Using a Nonpenetrating Chemical Probe, *p*-(Diazonium)-Benzenesulfonic Acid

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*Summary.* Spinach chloroplasts were exposed to $^{35}S$-labeled *p*-(diazonium)-benzenesulfonic acid (DABS), a water soluble compound which does not penetrate lipophilie regions of membranes, and which is highly reactive toward amino acid functiona-groups such as ε-amino, sulfhydryl, histidine, and tyrosine groups. Amino groups inl lipids can also form similar, stable covalent bonds by diazo coupling. Both chloroplast lipids and proteins were labeled with DABS, the total binding being about 1 DABS per 10 chlorophylls, depending on the reaction conditions.

After diazo coupling and subsequent digitonin fractionation into photosystems I and II enriched fractions, it was observed that PS-I was more highly labeled than PS-III usually by a factor of 10 to 24 times (on a per chlorophyll basis). After digitonin isolation, however, the PS-II portion bound an amount of DABS similar to the PS-I binding, We interpret these data as consistent with the binary membrane hypothesis (Arntzen. Dilley and Crane (1969), *J. Cell Biol.* **43**:16), which visualizes PS-I on the externa, "half" of a 90 Å grana membrane, and PS-II occurring on the interior "half" of thel membrane. The alternative explanation that PS-II and PS-I are arranged as a mosaic, and that the low DABS binding in PS-II is caused by burial of the diazo reactive groups in the interior of the proteins (and only exposed through the denaturing effect of digitonin) is not directly ruled out. However, this alternative is not consistent with the facts that: (a) most of the membrane proteins in PS-I and PS-II are identical in electrophoretic properties and therefore probably have similar overall structures; and (b) digitonin does not lead to appreciable denaturation of proteins, evidenced by the retention of PS-II electron transport activity.

Chloroplast thylakoid or inner membranes are highly differentiated in biochemical and structural aspects. Structurally, the grana, or stacked disc region contains two distinct types of particles visualized by the freeze etch technique (Mühlethaler, Moor & Szarkowski, 1965; Branton & Park,

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1961; Arntzen, Dilley & Crane, 1969). Based on Branton's experiments, Branton and Park (1967) postulated that the particles are embedded within the interior of the membrane; i.e., that the fracture plane goes along an interior layer rather than a layer on the exterior of the membrane (*cf.* Deamer & Branton, 1967). Wherli, Mühlethaler and Moor (1970) have agreed with this interpretation based on their double replica technique for freeze etching. Our work (*see* Arntzen *et al.*, 1969) and that of Park and Pfeilhofer (1969) agrees with Branton's viewpoint since surface adhering particles (coupling factor protein), visualized by negative staining, could be entirely removed from part of the thylakoids leaving them smooth by negative staining criteria (*cf.* Howell & Moudrianakis, 1967) without altering the appearance of the two particle fields seen by the freeze etch technique.

Digitonin fractionation of chloroplasts (Wessels, 1962; Anderson & Boardman, 1969) can result in the separation of two fractions, one enriched in photosystem II (oxygen evolution) activity and another, much lighter fraction enriched in photosystem I (NADP reduction) activity. Our earlier studies clearly showed that the PS-II enriched fraction was also enriched in the large 180 Å freeze etch particle, while the PS-I fraction was devoid of that particle but highly enriched in the smaller, 100 Å freeze etch particle (Arntzen *et al.*, 1969). Based on these findings plus additional thin section data we postulated that the chloroplast thylakoid membrane is a binary membrane with the PS-I localized on the outer "half" and PS-II localized within the inner "half" of a single 90 Å thick membrane. Briantais (1969) concluded that PS-I is on the outer portion of the membrane based on the sequential solubilization of PS-I and PS-II by Triton X-100. Later work by Jacobi and Lehmann (1969) and Sane, Goodchild and Park (1970) established that the stroma lamellae are devoid of both PS-II activity and the large freeze etch particles. The model published by Arntzen *et al.* (1969) thus refers to the grana lamellae membranes as regards the localization of photochemical activity.

This report deals with a technique for testing the above mentioned membrane model. The essence of the technique is to attach a water soluble chemical probe [*p*-(diazonium)-benzenesulfonic acid $^{+}N \equiv N - \langle\!\rangle - SO_3^-$ DABS] to thylakoid membranes, isolate PS-I and PS-II fractions by digitonin fractionation and measure the distribution of the DABS between the fractions. If the probe does not enter the lipophilic regions of the membrane and if PS-II is buried in the inner portion of the membrane, then more DABS should be found in the PS-I than in the PS-II fraction after digitonin fractionation. After fractionation and isolation, PS-II should also be readily labeled by the DABS.

Interpretation of the results depends on the validity of the assumption that DABS is very water soluble and not appreciably lipid soluble. Berg (1969) showed that DABS does not penetrate red blood cells, and that it reacts with externally exposed lipid and protein functional groups. The reactivity of diazonium compounds with phenolic, imidazole, ε-amino and α-amino, sulfhydryl, and guanidino groups of proteins has been documented by Howard and Wild (1957) and Higgins and Harrington (1959).

The results show that PS-I but not PS-II is readily labeled with DABS when applied to intact membranes, and that isolated PS-II after exposure to DABS becomes labeled to the same extent as PS-I.

## Materials and Methods

### *Preparation of Chloroplasts*

Since the diazonium salt would likely react with the amino groups of buffers such as Tris or Tricine, the chloroplasts were prepared in the usual way using 0.05 M phosphate buffer at pH 7.8 in 0.30 M NaCl. After washing in fresh grinding media and centrifugation, the pellet was resuspended in 0.1 M NaCl, 0.05 M $KPO_4$ pH 7.2 at about 3 mg chlorophyll (chl) per ml. The chlorophyll assays were according to the method of Arnon (1949).

### *Preparation of $^{35}$S-DABS*

The diazotization of $^{35}$S-*p*-amino benzenesulfonic acid (also known as sulfanilic acid and obtained from New England Nuclear, 3.94 mC/mmole) was carried out essentially as described by Hoyer and Trebold (1970). 120 mg $^{35}$S-sulfanilic acid and 70 mg unlabeled sulfanilic acid (a total of 1 mmole) were dissolved in 4 ml 0.37 M KCl (to aid in solubilizing the sulfanilic acid). After cooling to ice bath temperature and adding 4 ml of 1 M cold HCl, 76 mg $NaNO_2$ (1.1 mmole) in 2 ml cold water was added with stirring. The mixture was allowed to stand in the ice bath for 20 min, at which time 5 mg sulfamic acid ($NH_2SO_2OH$) was added to neutralize the excess $NaNO_2$. The starch iodide paper test was still slightly positive, but after adding 6.7 ml 0.15 M $K_2HPO_4$ and bringing the pH to 7.5, the starch iodide test paper remained colorless after wetting. The mixture was brought to 50 ml (0.02 M DABS final concentration) and 5 ml aliquots were frozen for later use. The specific activity of the $^{35}$S-DABS was $4 \times 10^5$ cpm/μmole. The $\beta$ emission from $^{35}$S was counted in an end window Geiger counter.

Unlabeled DABS was prepared in a similar manner except no sulfur labeled sulfanilic acid was used.

### *Coupling $^{35}$S-DABS to Chloroplast Membranes*

Generally, 0.4 mM $^{35}$S-DABS (final concn) was reacted with chloroplasts having 1 mg chl/ml in 0.1 M NaCl, 0.05 M $KPO_4$ (saline $PO_4$) at ice bath temperature. Deviations from these conditions are listed in the table legends. After a reaction time varying from 30 sec to 15 min, the solution was diluted with cold saline-$PO_4$, centrifuged at 15,000 × *g* for 15 min. The chloroplast pellet was resuspended in 30 ml saline-$PO_4$, homogenized and centrifuged again. This washing procedure was repeated once or twice more (two

saline-$PO_4$ washes after the initial sedimenting out of the reaction mixture, removed more than 98% of the unreacted DABS). The final pellet was resuspended in 0.01 M KCl, 0.05 M $KPO_4$ to 0.25 mg chl/ml in preparation for digitonin fractionation or 0.15 M KCl, 0.05 M $KPO_4$ to 0.5 mg chl/ml for French Press treatment.

## *Digitonin Fractionation and Biochemical Assays*

The diazotized chloroplasts in 0.01 M KCl, 0.05 M $KPO_4$ at 0.25 mg chl/ml were added (3 parts) to 1 part 2.0% (w/v) twice recrystallized digitonin (Sigma) and stirred for 30 min at 15 °C. The mixture (5 ml) was then loaded onto sucrose step gradient tubes having the following steps: bottom, 15 ml 1.8 M sucrose; middle, 10 ml 1.0 M sucrose; top, 9 ml 0.7 M sucrose with 0.5% digitonin to keep PS-I and PS-II from re-aggregating. (All sucrose solutions were 0.05 M $KPO_4$ pH 7.2.) Centrifugation was 60 min at 27,000 rpm in a Spinco SW-27 rotor. The two major bands corresponding to PS-I (top) and PS-II (bottom) were separately removed and assayed for chlorophyll, radioactivity and photochemical activity.

The PS-I activity was measured spectrophotometrically at 340 nm by the photochemical reduction of NADP using ascorbic acid (AA) and dichlorophenol indophenol (DPIP) as the donor system. The assay consisted of: 50 mM $K_2HPO_4$, pH 6.8, 7.5 mM AA, 0.16 mM DPIP, 0.37 mM NADP, and usually 20 µg chl/ml. Saturating amounts of plastocyanin, ferredoxin (Fd) and Fd-NADP reductase were added.

PS-II activity was measured by following the reduction of DPIP spectrophotometrically at 580 nm, using diphenylcarbazide as the electron donor substituting for water (*cf.* Vernon, Shaw & Ke, 1966; and Vernon & Shaw, 1969 for details about the assays).

## *Separation of Grana from Stroma Lamellae*

For some experiments, following diazo coupling, the stroma membranes were removed from the grana stacks by French Press treatment (4,000 psi) followed by differential centrifugation (Sane *et al.*, 1970). This permitted a comparison of DABS binding to stroma and grana lamellae. The grana fraction was further fractionated by digitonin and PS-I and PS-II fractions were purified according to Arntzen, Dilley, Peters and Shaw, 1971, and assays run for chlorophyll content, radioactivity, and photochemical activity.

## *Electron Microscopy of Chloroplast Membranes*

The distribution of DABS between digitonin PS-I and PS-II fractions was tested on chloroplasts suspended in the usual high salt (0.1 M NaCl − 50 mM $PO_4$) and a low salt medium (0.1 M sucrose was used to resuspend a pellet centrifuged out of a chloroplast suspension having 0.1 M sucrose, 6.7 mM NaCl, and 3.3 mM $PO_4$). The salt concentration in the sucrose resuspended chloroplasts was diluted approximately 10-fold or more, giving a final salt concentration of less than 1 mM.

The purpose of this experiment was to test whether chloroplasts having a tightly stacked grana configuration bound less DABS than chloroplasts expanded in low salt. Izawa and Good (1966) first demonstrated that low salt-suspended chloroplasts lose their grana stacking. To control and low salt chloroplasts, having 5.5 mg chlorophyll, was added 0.4 mM final concentration of $^{35}$S-DABS (giving at most 3 mM total salts in 5 ml total volume). After 2 min of incubation, glutaraldehyde to 2% final concentration was added to a 2-ml aliquot of each sample, the remainder being carried through the routine outlined above for digitonin fractionation.

The chloroplast samples were further fixed in osmium tetraoxide, dehydrated, embedded in Epon, thin-sectioned, and post-stained with uranyl nitrate and lead citrate in the usual manner (Arntzen *et al.*, 1969). Electron micrographs were taken with a Phillips 300 microscope.

## Results

### *Evidence for $^{35}S$-DABS Binding*

That coupling occurred between the $^{35}S$-diazosulfanilic acid and some functional groups on the chloroplast membrane, is shown by the fact that $^{35}S$ isotope was firmly bound to the membrane fraction and did not wash off after three washes with NaCl-$PO_4$ solution. A typical experiment resulted in the binding of 1 DABS per 8 chlorophylls (Table 1, Part A).

Table 1. $^{35}S$-DABS binding to chloroplast membrane lipid and protein fractions

| A. CPM Observed in 0.025 ml | CPM in total fraction (2.2 ml) | μmoles DABS in total fraction (sp act $=4.0 \times 10^5$ cpm per μmole) | Total chl in fraction (μmole) | chl/DABS |
|---|---|---|---|---|
| 3,500 cpm | $3.1 \times 10^5$ | 0.77 | 6.6 | 8.6 |

Sample calculation of DABS content. Chloroplasts prepared as described in Materials and Methods having 5 mg chl were suspended in 5 ml 0.1 M NaCl, 0.05 M $KPO_4$ pH 7.2 at ice temperature. To this was added 0.5 ml of 0.02 M $^{35}S$-DABS (sp act $4 \times 10^5$ cpm/μmole of DABS). After reacting 15 min at ice bath temperature, the mixture was diluted with saline-$PO_4$ and centrifuged at $15{,}000 \times g$ for 15 min, resuspended in 30 ml 0.1 M NaCl, 0.05 M $KPO_4$ pH 7.2 and centrifuged again. This washing step was repeated 3 more times to wash out all unreacted $^{35}S$-DABS. The final pellet was resuspended in 2.2 ml 0.01 M KCl, 0.05 M $KPO_4$ pH 7.2 and an aliquot plated for counting radioactivity.

| B. Fraction | CPM Recovered | % Distribution |
|---|---|---|
| First acetone wash | $490 \times 10^3$ | 45% |
| Second acetone wash | $24 \times 10^3$ | 2% |
| Third acetone wash | $8 \times 10^3$ | 1% |
| Fourth acetone wash | $11 \times 10^3$ | 1% |
| $ChCl_3$/MeOH wash | $100 \times 10^3$ | 10% |
| Protein residue | $460 \times 10^3$ | 40% |

Another chloroplast sample was treated with $^{35}S$-DABS in the same manner and the resultant washed chloroplasts were extracted with four successive 30-ml aliquots of acetone (100%), the membranes being centrifuged out of the acetone each time. A fifth wash of 30 ml 3:1 chloroform/methanol was then given. Aliquots of each organic phase and of the final resuspended protein pellet were counted for radioactivity, and the total cpm for each fraction were calculated.

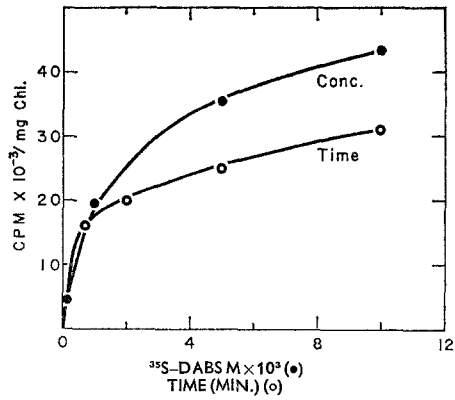


Fig. 1. Concentration curve and time course for DABS binding to chloroplast membranes. Concentration curve experiment: Chloroplasts were treated as described in Part A of Table 1 except that the reaction time was 5 min after adding the indicated concentration of $^{35}$S-DABS. The concentrations should be corrected to 0.4 the values given on the abscissa. Time course experiment: Conditions similar to Part A, Table 1 except that 0.4 mM $^{35}$S-DABS was used for the time periods indicated prior to beginning centrifugation. For both experiments, the ordinate gives the counts per min bound to the chloroplasts after 3 washings with saline-$PO_4$

After two washings, the pellet contained $3.3 \times 10^5$ cpm and the last 30 ml supernatant contained $3 \times 10^4$ cpm (0.6% of the counts originally added). Further washing did not remove a significant amount of label from the membranes. The actual data used to compute the level of DABS binding is shown in Table 1, part A.

To test the possibility that DABS is partly held on the membrane by electrostatic rather than covalent binding, an experiment was carried out using a washing step having unlabeled DABS present. After a $^{35}$S-DABS reaction similar to that given in Table 1, part A, all four aliquots of treated chloroplasts were centrifuged and the pellets resuspended in 5 ml of the fresh NaCl-$PO_4$ washing media. To two of the four tubes, unlabeled DABS was added to a final concentration of 4 mM, twice the concentration of $^{35}$S-labeled DABS used for the labeling step. After a 2-min incubation to allow any exchange to occur, the suspensions were centrifuged and washed with NaCl-$PO_4$ solution three times. The first supernatants after the unlabeled DABS treatment were kept for isotope counting as were the final pellets. The data showed no significant difference in the number of counts either in the supernatant of the first wash after unlabeled DABS was given to two of the four tubes (300 cpm/ml supernatant in the tubes not receiving unlabeled DABS and 330 cpm/ml supernatant in the tubes given unlabeled DABS), or in the final resuspended pellets (7,400 cpm total bound DABS

and 7,800 cpm total DABS, respectively). The standard deviation in this latter experiment was ±350 cpm, about the same value as the observed difference. The observed difference, while not statistically significant is in the wrong direction for the data to suggest exchange of cold DABS for labeled DABS. Therefore, the DABS must be bound by covalent linkages, as expected.

A second test for the noncovalent binding of the labeled compound or a derivative was conducted by exposing chloroplasts to $^{35}$S-*p*-amino benzene sulfonic acid (not having the diazo group). If electrostatic binding or lipid solubilization were significant, then one might expect to find this compound in the PS-I fraction. This experiment resulted in the binding of 1 sulfanilic acid per 210 chlorophylls in the PS-I fraction, about 5% as much binding as normally occurred with DABS under those conditions. This value is very close to the statistical variation of this particular experiment, being 2.5 cpm above background. From 200 to 5,000 cpm were counted in the planchettes in most of the $^{35}$S-DABS experiments, depending on the particular experiment. The small number of counts which occurred in the PS-I fraction were removed by a washing step using unlabeled sulfanilic acid. In that experiment the counts were exactly the same as the background, i.e., 23 cpm.

Fig. 1 shows the effect of DABS concentration on binding and the time course of binding. A concentration of 4 mM DABS is nearly saturating for a 5 min reaction time. The time course data shows that the coupling reaction is more than 50% complete by the end of 1 min when 0.4 mM DABS is used.

### *DABS Binding to Lipid and Protein Fractions*

Chloroplasts were treated with $^{35}$S-DABS at 5 mM for 15 min and the unreacted DABS removed by washing. Lipids were totally extracted by four 100% acetone extractions and one chloroform/methanol (3:1) extraction. Part B of Table 1 shows the distribution of $^{35}$S-DABS in the lipid extracts and the remaining protein fraction. Nearly 60% of the DABS is bound to lipids (or acetone extractable lipoproteins) with 40% remaining bound to the protein fraction.

### *DABS Binding to PS-I and PS-II Fractions*

The above results indicate that DABS binding can be used as a marker of chloroplast membrane lipids and proteins. It was of interest to determine the relative binding of this water soluble, highly charged marker between PS-I and PS-II fractions. For this purpose chloroplasts were reacted with $^{35}$S-DABS, washed free of unreacted DABS and fractionated with digitonin.

Table 2. $^{35}$S-DABS labeling in PS-I and PS-II

| | Fraction | μmole chl / μmole DABS | Ratio of DABS in PS-I to DABS in PS-II | Photochemical activities μmole (hr · mg · chl)$^{-1}$ | |
|---|---|---|---|---|---|
| | | | | PS-II Assay | PS-I Assay |
| | | | | DPC → DPIP | AA + DPIP → NADP |
| A. | PS-I | 8.4 | 13 | 31 | 205 |
| | PS-II | 112 | | 145 | 16 |
| B. | PS-I | 21 | 24 | | |
| | PS-II | 496 | | | |
| C. | PS-I | 38 | 10 | 15 | 300 |
| | PS-II | 400 | | 125 | 12 |

Part A. Chloroplasts were treated with $^{35}$S-DABS as described under Table 1. After washing the unreacted DABS away from the chloroplasts by three washings and centrifugations, the final pellet was resuspended in 0.01 M KCl, 0.05 M $KPO_4$ pH 7.2 and adjusted to 0.25 mg chl/ml. Three parts of this material were mixed with 1 part 2% digitonin, incubated 30 min at 15 °C with constant stirring. The incubated chloroplasts were loaded onto step gradient tubes as described under Materials and Methods and centrifuged 1 hr. Aliquots of the PS-I and PS-II fractions were taken for photochemical activity assays.

Part B. The chloroplasts were diazotized and digitonin incubated as in Part A above, except the exposure time to $^{35}$S-DABS was limited to the time required to add the $^{35}$S-DABS to the chloroplasts, stir 3 to 4 sec and immediately begin centrifugation. The DABS concentration was lowered to 0.4 mM.

Part C. Treatment similar to part B except that 50 mg of albumin were added at 1 min to react with any unreacted DABS, followed by immediate centrifugation.

The PS-I and PS-II fractions were separated by a density gradient technique similar to that of Ohki and Takamiya (*see* Materials and Methods). The PS-I and PS-II fractions were assayed for radioactivity, chlorophyll concentration, and PS-I and PS-II photochemical activity. Table 2 shows some typical results, indicating that the PS-I fraction is more highly labeled by DABS than PS-II. On a chlorophyll basis, PS-I contained from 10 to 24 times more DABS than the PS-II fraction. The variability in the amount of DABS bound by the fractions is accounted for by the shorter exposure time and lower DABS concentration in experiments B and C compared to experiment A. Albumin was added to the diazo coupling reaction mixture of experiment C with the intention of rapidly reacting with unreacted DABS. The treatment did not alter the usual pattern of PS-I and PS-II fractionation as shown by Table 2. As is usual, there is some residual PS-I activity in the PS-II fraction (Arntzen *et al.*, 1971).

Apart from the hypothesis being tested of nonexposure to the bathing solution, is there any inherent reason for the PS-II fraction not to couple with DABS? To test this, the isolated PS-II was mixed with fresh DABS,

Table 3. $^{35}$S-DABS labeling of PS-II after digitonin isolation

| Conditions | $^{35}$S-DABS binding to PS-II fraction (μmole chl/μmole DABS) |
|---|---|
| 1. DABS reaction with intact membranes (before digitonin treatment) | 400 |
| 2. DABS reaction with digitonin isolated PS-II fragments | 22 |

Part of the PS-II fraction from Part C of Table 2 was centrifuged out of the sucrose medium and resuspended in 0.1 M NaCl, 0.05 M $KPO_4$. This fraction was then reacted with $^{35}$S-DABS as described in Part B of Table 2. After washing away unreacted DABS by three washings (which removes > 98% of removeable counts), the pellet was resuspended and aliquots were counted for radioactivity and assayed for chlorophyll.

and thereafter treated similarly to the chloroplasts used for the Table 1 data. Table 3 shows that whereas the PS-II fraction bound only 1 DABS per 400 chlorophylls when the membranes were intact, the isolated PS-II bound 1 DABS per 22 chlorophylls, about the same level of binding as PS-I (*see* Part B, Table 2).

### *DABS Binding to Stroma vs. Grana Membranes*

Chloroplasts labeled with $^{35}$S-DABS were passed through a French Press four times to remove stroma lamellae from grana stacks (Sane *et al.*, 1970; Arntzen *et al.*, 1971). Table 4 shows that the stroma fraction contained 1 DABS per 2.6 chlorophylls compared to 1 DABS per 20 chlorophylls in the grana fraction. The photochemical assays indicate that the stroma fraction was quite clean, having low PS-II and high PS-I activity.

The grana portion obtained in this experiment was fractionated with digitonin and the DABS labeling was assayed in each fraction. Table 4 shows again that the PS-I fraction was more highly labeled than the PS-II fraction by a 6:1 ratio. The photochemical assays indicated that a usual PS-I and PS-II split of the grana occurred (Arntzen *et al.*, 1971).

A variation on the above experiment involved treating the isolated grana fraction with DABS after the stroma membranes were removed. In this case [Table 4, Part B(2)] the distribution of DABS labeling in the PS-I and PS-II fractions was 20 and 317 chl/DABS, respectively.

### *DABS Binding to Low Salt-Suspended Chloroplasts*

There is a possibility that the lower level of PS-II binding of the diazo compound is caused by the inability of DABS to penetrate the partition

Table 4. $^{35}$S-DABS binding to chloroplast stroma and grana

| A. | $^{35}$S-DABS bound μmole chl/μmole DABS | Photochemical activities μmole (hr · mg · chl)$^{-1}$ | |
|---|---|---|---|
| | | PS-II assay | PS-I assay |
| | | DPC → DPIP | AA + DPIP → NADP |
| Stroma | 2.6 | 10 | 124 |
| Grana | 20 | | |
| Chloroplasts prior to French Press treatment | 14 | | |

Part A. *$^{35}$S-DABS binding in stroma and grana.* Chloroplasts were exposed to $^{35}$S-DABS and washed free of unreacted label as given in Part B, Table 2. The washed membranes were resuspended in 0.15 M KCl, 0.05 M $KPO_4$ pH 7.2 and passed through a French Pressure Cell four times as described in Materials and Methods. The stroma fractions were combined (the two 40,000 × *g* centrifugation supernatants) and aliquots were taken for radioactivity counting, chlorophyll assays, and PS-I and PS-II photochemical assays.

| | Fraction | $^{35}$S-DABS bound μmoles chl/μmole DABS | PS-II assay | PS-I assay |
|---|---|---|---|---|
| B. (1.) | Grana PS-I | 48 | 26 | 268 |
| | Grana PS-II | 292 | 79 | 0 |
| (2.) | Grana PS-I | 20 | 13 | 300 |
| | Grana PS-II | 317 | 61 | 0 |

Part B. *Distribution of DABS within the grana fraction.* (1.) The final 40,000 × *g* pellet taken up in 0.10 M KCl, 0.05 M $KPO_4$ pH 7.2 representing the grana fraction, was assayed for radioactivity and chlorophyll content and the balance of the material was fractionated with digitonin as described in Part A of Table 2. The PS-I and PS-II fractions obtained from this grana preparation were assayed for radioactivity, chlorophyll content, and PS-I and PS-II photochemical activity. (2.) In this experiment the grana fraction was purified away from the stroma lamellae as above and then subjected to the coupling step as given in Part B, Table 2.

regions of grana stacks. To test this, chloroplasts were first suspended in low salt (less than 3 mM total salt) and 0.1 M sucrose prior to exposure to 0.4 mM $^{35}$S-DABS. Izawa and Good (1966) and Anderson and Vernon (1967) have shown that such treatment leads to chloroplast grana becoming unstacked, with the formation of separated lamellae. Aliquots of control and low salt-treated chloroplasts were prepared for electron microscopy as described under Materials and Methods and the remainder treated in the usual way for digitonin fraction. Table 5 gives the results of the DABS

Table 5. DABS binding to chloroplasts suspended in low salt

| Sample | chl/DABS | |
|---|---|---|
| | PS-I | PS-II |
| High salt control | 23 | 320 |
| Low salt | 32 | 440 |

Chloroplasts were washed free of the NaCl-$PO_4$ suspending medium by diluting with sucrose and centrifuging. The pellet was suspended in salt-free sucrose. Control chloroplasts were kept in 0.1 M NaCl–50 mM $PO_4$.

Control and low salt-suspended chloroplasts having 5.5 mg chl in 5 ml total volume were exposed to 0.4 mM $^{35}$S-DABS for 2 min. We treated 2 ml of each with glutaraldehyde and prepared them for thin-sectioning as described in Materials and Methods; the remainder was diluted with NaCl–$PO_4$ and prepared for digitonin fractionation as given in Table 2.

labeling, showing somewhat less DABS binding in both PS-I and PS-II in the low salt chloroplasts compared to the high salt controls. Fig. 2 shows the ultrastructural appearance of the control chloroplasts after 2 min of 0.4 mM DABS exposure. Fig. 3 shows the appearance of the low salt chloroplasts after a similar DABS treatment.

## Discussion

The data show that diazosulfanilic acid readily couples to some groups on lipid and protein molecules of chloroplast thylakoid membranes. Fig. 1 shows that the coupling reaction is about 80% complete in 2 min, and that 2 mM DABS is at nearly saturating concentration under the conditions used here. Control experiments established that two washings of the DABS treated membranes, after an initial dilution and centrifugation of the reaction mixture, were sufficient to remove all but traces of unbound DABS. A third wash removed about 0.6% of label originally added, and some of this may have been bound to proteins extracted from the membranes into the saline-$PO_4$ washing solution. That a covalent rather than an electrostatic binding occurred, was indicated by the fact that washing the $^{35}$S-DABS reacted membranes with cold DABS did not remove a significant number of counts from the membranes. Also, there was no significant binding of $^{35}$S-sulfanilic to the PS-I fraction, suggesting that the diazo group was essential for significant binding.

Both lipid and protein portions of the chloroplast membranes react with DABS (Table 1), binding about 60 and 40%, respectively, of the total DABS bound. At this time, there is no data on which lipids or proteins react with the diazosulfanilic acid. Gel electrophoresis of PS-I and PS-II

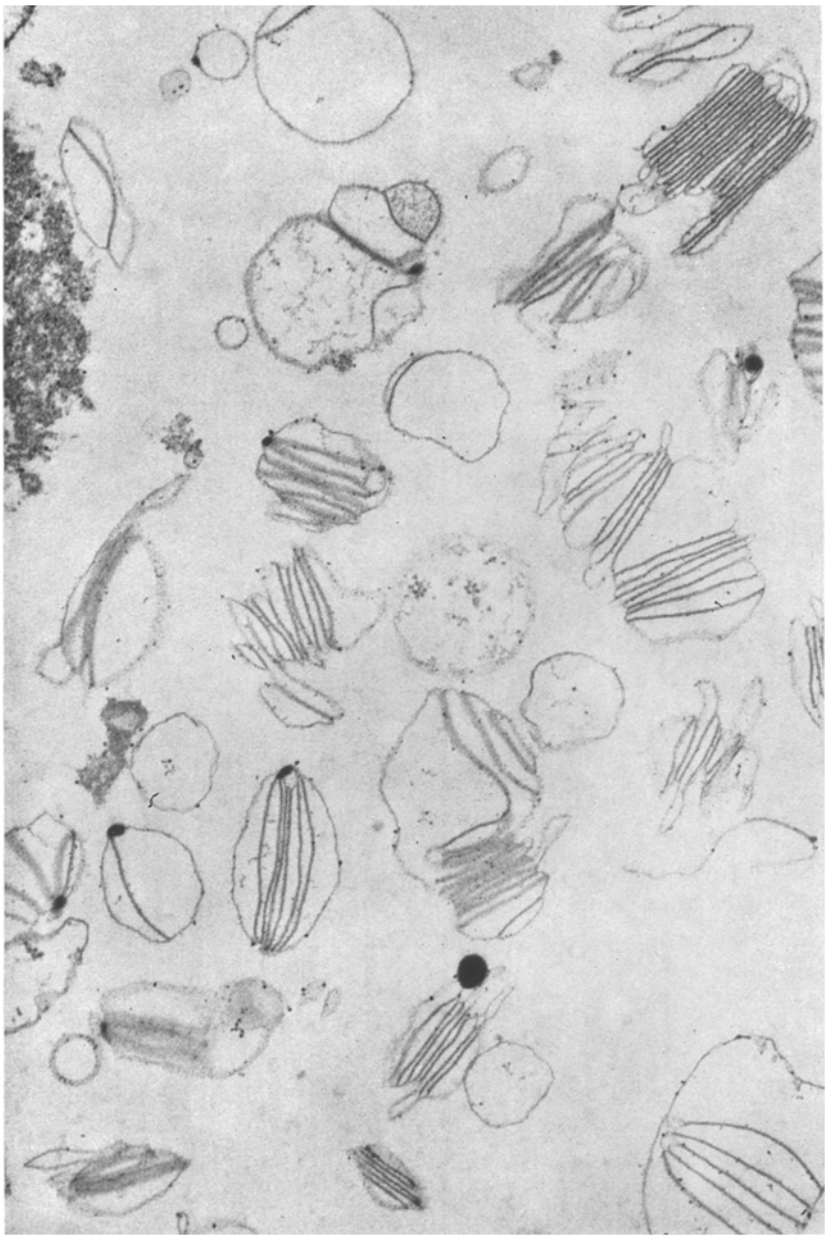

Fig. 2. Ultrastructure of control chloroplasts kept in 0.1 M NaCl–50 mM $PO_4$. The chloroplasts were treated with $^{35}$S-DABS as described in Table 5. 24,300 ×

proteins (Arntzen, 1970; Crane, Arntzen, Hall, Ruzicka & Dilley, 1970) and lipid analysis (F. Allen, *personal communication*) indicate that most of the protein bands are common to both systems and the lipid constituents

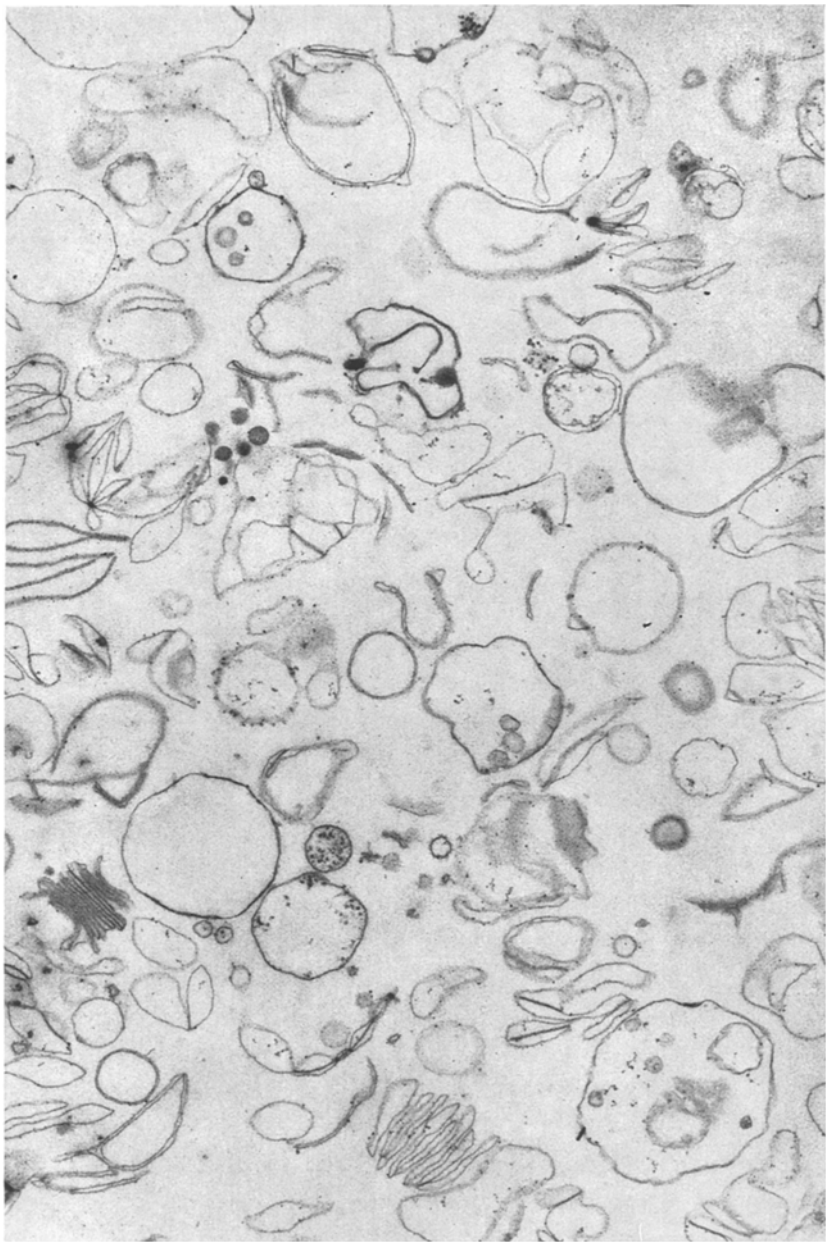

Fig. 3. Ultrastructure of low salt-suspended chloroplasts after $^{35}$S-DABS treatment. 24,300 ×

are generally similar. Therefore, it is logical that both PS-I and PS-II reactable protein and lipid groups would react with DABS to a similar extent given equal accessibility.

$^{35}$S-DABS, a water soluble, nonpenetrating diazo compound is bound to the PS-I fraction at a concentration 10 to 24 times greater (on a chlorophyll basis) than bound to the PS-II portion when the DABS is reacted with intact chloroplast thylakoids (Table 2). However, when PS-II is isolated from the membrane and then exposed to the DABS, it becomes labeled to about the same extent at the PS-I fraction (Table 3). Diazo coupling would not be expected to occur with the digitonin which probably remains in the PS-II fraction. Digitonin has aliphatic type hydroxyl groups in the ring which are not appreciably reactive in diazo coupling (Brewster, 1948). The similar level of DABS labeling in exposed PS-II compared to PS-I under similar conditions also indicates that groups on chloroplast lipids and proteins react in both PS-I and PS-II particles.

The same pattern of DABS binding was found when isolated grana stacks were fractionated with digitonin (Table 4). When the DABS treatment preceded the French Press step, the distribution of DABS in the grana PS-I and PS-II was 6:1 [Table 4, Part B(1)], compared to a ratio of 15:1 [Table 4, Part B(2)] when the diazo coupling step was given to the purified grana fraction. French Press treatment causes the loss of membrane bound components such as plastocyanin (Arntzen *et al.*, 1971; Baszynski, Brand, Krogmann & Crane, 1971) and perhaps other proteins or lipoproteins. This probably accounts for the lower ratio of DABS in PS-I compared to PS-II in the grana preparation treated with DABS prior to French Press disruption. The similar level of DABS binding in the two PS-II preparations [Table 4, Parts B(1) and (2)] supports this viewpoint, the difference being attributable to the PS-I fraction.

In every experiment we noticed that digitonin treatment removed a significant amount of DABS so that it was not recovered with the chlorophyll containing membranes. Probably this is because the detergent solubilizes some of the membrane lipid. It was found that 60% of the total DABS binds to a lipid extractable fraction (Table 1). The loss of membrane lipid by digitonin solubilization could explain why the isolated grana stacks were labeled at a level more than twice that of the PS-I subsequently isolated from the grana (Table 4).

Table 4 shows that the stroma fraction was more highly labeled than the grana fraction (2.6 chl/DABS compared to 20 chl/DABS, respectively). An unknown portion of the counts recovered in the stroma fraction are very likely associated with nonmembrane or loosely adhering proteins not intimately associated with the PS-I or PS-II membrane structures. In addition to plastocyanin released by French Press treatment, we have routinely observed a high level of extraneous protein in the $144{,}000 \times g$

stroma pellet (G. A. Peters, C. J. Arntzen and R. A. Dilley, *unpublished observations*). Some of these proteins (such as coupling factor protein) could have initially been associated with the grana region. In any event, the PS-I fraction in all cases is more highly labeled than the PS-II portion, even though the PS-II fraction when separated from the PS-I fraction is capable of the same level of DABS coupling.

The separation of PS-I and PS-II by digitonin after DABS treatment was similar to that obtained routinely with spinach chloroplasts in our hands (Arntzen *et al.*, 1971). Diazo coupling did not appreciably affect the split nor the photochemical activities as far as we could tell.

It has routinely been observed that the grana PS-II fraction is "contaminated" with a certain amount of PS-I photochemical activity and P700 chlorophyll (Arntzen *et al.*, 1971). The electron transport assays shown in Tables 2 and 4 demonstrate this. Assuming that PS-I is entirely localized on the outer "half" of the grana membrane, one could account for the DABS found in the PS-II fraction after digitonin treatment as due to that bound to the PS-I fragments adhering to the PS-II fraction. Using a similar argument based on electron transport activities and P700 content, Arntzen *et al.* (1971) were able to entirely account for the PS-I activity in the PS-II fraction as due to contaminating P700 (PS-I). This cannot be taken as an absolute argument until complete separation of PS-I activity (and P700) from the PS-II fraction is accomplished, but it is a point in favor of the interpretation we place on these data.

It is also a possibility that the large 180 Å freeze etch particles, which occur on the inner PS-II portion of the membrane, protrude into the outer PS-I portion far enough to have a small amount of that structure very close to the external surface of the membrane. If this is the case, there would be a greater likelihood of a compound such as DABS being able to react with the lipid or protein of that small "tip" than reacting with the majority of the PS-II portion which is masked by the PS-I portion.

Whether this situation occurs cannot be answered at this time, but regardless, the DABS labeling indicates that PS-I is considerably more available to the external phase than is the bulk of the PS-II portion of the membrane.

The experiment designed to test whether the low PS-II DABS binding could be caused by nonpenetration of DABS into the appressed grana partitions, showed that DABS binding was somewhat less in the low salt treatment (designed to unstack the grana) than in the high salt control (Table 5). The electron micrographs show that the low salt-treated chloroplasts had mostly extended lamellae with little grana stacking, while the

control chloroplasts were considerably disrupted relative to chloroplasts *in situ*, but they still retained a considerable degree of appressed grana discs (Figs. 2 and 3). Apparently, the degree of appressed partitions did not significantly affect the penetration of DABS along the membrane surfaces. If the partitions in stacked regions had kept DABS out of those regions, we should have found much higher DABS binding in the low salt chloroplasts since there were fewer partitions. There is no evidence that the partitions exclude solutes. Our recent work with digitonin fractionation of isolated grana stacks indicates that approximately 50% of the total chlorophyll is associated with each photosystem since they are separable by digitonin fractionation (Arntzen *et al.*, 1971). If this is the case, then the experiments described by Table 5 and Figs. 2 and 3 are best explained by assuming that the PS-I apparatus is equally accessible to solutes whether it is in the end membrane or in the appressed regions.

These data are consistent with the binary membrane model of Arntzen *et al.* (1969) in which it was proposed that the PS-I apparatus comprises the outer half and the PS-II apparatus comprises the inner half of a 100 Å chloroplast membrane. Fig. 4 shows this model as previously published.

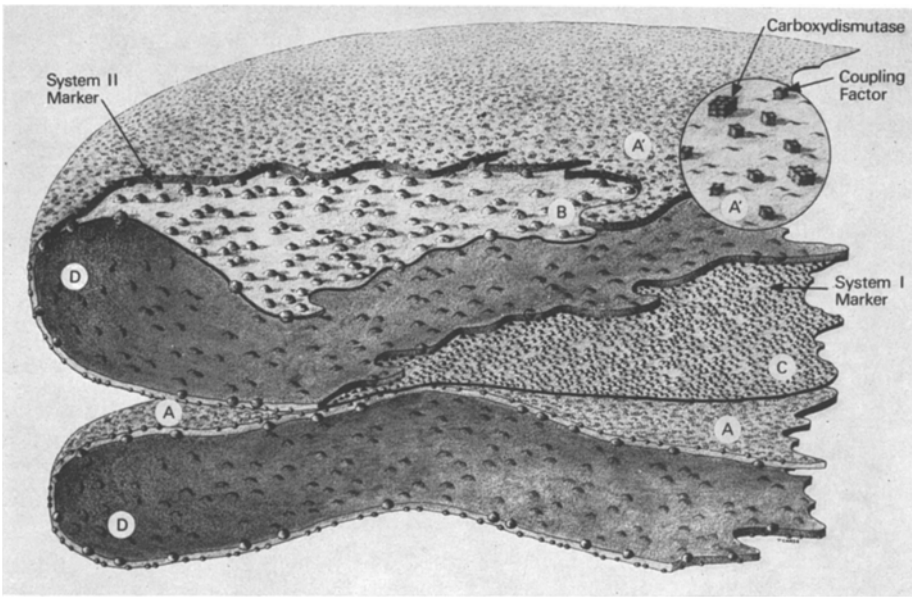


Fig. 4. An artist's rendition of the chloroplast membrane model proposed earlier by Arntzen *et al.* (1969). This model is supported by the present work. The binary membrane feature is thought to be valid for both grana and stroma regions, but the stroma region has a 110 Å size widely dispersed freeze etch particle (here identified as a PS-II marker) rather than the 180 Å sized particle and no PS-II photochemical activity

Since it is now known that the stroma lamellae have little or no PS-II activity (Jacobi & Lehmann, 1969; Sane *et al.*, 1970; Arntzen *et al.*, 1971), and no large freeze etch particles (Sane *et al.*, 1970), the model is to be related to the grana region as regards biochemical function.

These data do not absolutely rule out an alternative arrangement of PS-I and PS-II portions in which they may be visualized as arranged in a mosaic with PS-II having essentially all of its diazo-reactive groups buried either beneath a lipid barrier or buried within the interior of the PS-II proteins. The rationale for greater reactivity of PS-II toward the DABS after digitonin isolation could be that the lipid barrier is removed or the proteins of PS-II are partly denatured, thus exposing reactable groups. The latter seems improbable since neither digitonin nor DABS treatment results in a significant reduction in electron transport activity, a fact inconsistent with denaturation of PS-II proteins by the treatment. The former possibility that the PS-II lipids mask diazo-reactive groups which are exposed by digitonin treatment is somewhat unreasonable in view of the fact that both PS-I and PS-II digitonin fractions have generally similar lipid composition (F. Allen, *personal communication*). Therefore, we feel that these data are reasonably interpreted as consistent with the binary membrane model.

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