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LIGHT-INDUCED CHANGES IN RESISTIVITY OF SPINACH CHLOROPLASTS TOWARD MODIFICATION WITH DIAZONIUM-1,2,4-TRIAZOLE

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SUMMARY

Spinach chloroplasts in the light and in the dark were treated with several reagents for protein modification to see the effect of light on their resistivity toward modification. The reagents were *p*-diazobenzenesulfonic acid, diazonium-1-H-tetrazole, sodium-2,4,6-trinitrobenzenesulfonate, sodium-β-naphtoquinone-4,6-disulfonate and diazonium-1,2,4-triazole. No difference in the absorption spectrum was found between chloroplasts treated with these reagents in the light and those treated in the dark. However, these light- and dark-treated samples when solubilized with a nonionic detergent showed a difference in turbidity. Diazonium-1,2,4-triazole was the most suitable of the above reagents, and the solubilized sample of chloroplasts treated with diazonium-1,2,4-triazole in the light showed a turbidity which was about 2-fold higher than that of the same sample treated in the dark. This increase in turbidity was interpreted as being due to a change in the resistivity toward chemical modification of chloroplasts caused by illumination. In the presence of 3-(*p*-chlorophenyl)-1,1-dimethylurea, pentachlorophenol and 2-methylthio-4,6-bis-isopropylamino-*s*-triazine, which are inhibitors of the Hill reaction, the light-induced increase of turbidity was suppressed by 72, 78 and 62 %, respectively. The addition of ATP caused a much greater increase of turbidity both in the light and in the dark. It was thus found that light and ATP induce a configurational change of chloroplasts or a conformational change of chloroplast proteins inside.

INTRODUCTION

Structural changes of chloroplasts have been observed by various workers. PACKER[1] found a light-induced change of 90° in light-scattering by chloroplasts. ITOH *et al.*[2] and YAMASHITA *et al.*[3] demonstrated light-induced shrinkage of chloroplasts in phosphate buffer from the volume changes measured with a Coulter counter and from changes in cross-sectional area measured by light-scattering, light-absorption efficiency and electron microscopy. NISHIDA[4] measured this light-induced shrinkage with phosphates and the swelling of chloroplasts without phosphates by means of

Abbreviations: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate.

a hematocrit. Another light-dependent process related to these structural changes of chloroplasts is the uptake of calcium and phosphate[5] and efflux of potassium upon illumination, and the light-induced pH increase of chloroplast suspensions found by JAGENDORF AND HIND[7]. Recently MURAKAMI AND PACKER[8] have observed by electron microscopy that the thickness and spacing of thylakoid membranes decrease on illumination.

In the present study, the effect of light on the resistivity of chloroplasts toward modification with protein reagents was examined as a new approach for studying this interesting phenomenon of shrinkage and swelling of chloroplasts. The reagents examined were *p*-diazobenzenesulfonic acid[9], diazonium-1-H-tetrazole[10] and diazonium-1,2,4-triazole for modification of histidine and tyrosine residues and sodium 2,4,6-trinitrobenzenesulfonate[11] and sodium β-naphtoquinone-4,6-disulfonate[12] for amino groups. The chloroplasts treated with these reagents in the light and those treated in the dark were solubilized with a nonionic detergent, Emulgen, and the resultant suspensions were measured by turbidimetry. Not much difference in the absorption and turbidity spectra was found between the light- and dark-treated samples before solubilization.

EXPERIMENTAL

Reagents

Diazonium-1,2,4-triazole was prepared by essentially the same procedure as described for the preparation of diazonium-1-H-tetrazole[10]. 200 mg of 3-amino-1,2,4-triazole were dissolved in 23 ml of a 1.6 M HCl solution and diazotized in an icebath by the addition of $NaNO_2$ in 10 ml of water. This solution of diazonium-1,2,4-triazole was adjusted to pH 3.5 with KOH before being mixed with a chloroplast suspension. The molar concentration of diazonium-1,2,4-triazole was determined, assuming that all of 3-amino-1,2,4-triazole dissolved was completely diazotized into diazonium-1,2,4-triazole. Diazonium-1-H-tetrazole and *p*-diazobenzenesulfonic acid were prepared by the method of HORINISHI *et al.*[10]. Sodium 2,4,6-trinitrobenzenesulfonate was obtained from Tokyo Kogyo Co. Sodium β-naphtoquinone-4,6-disulfonate was purchased from Seikagaku Kogyo Co. Emulgen-810 was kindly donated by Kao-Atlas Co.

Preparation of chloroplasts

Whole chloroplasts were isolated from leaves of *Spinacia oleracea* (spinach) in the following manner as described by OGAWA *et al.*[13]. The procedure includes squeezing chloroplasts out of leaves through cotton cloth into 0.04 M phosphate buffer (pH 7.2). The green juice was centrifuged in a refrigerated centrifuge at 100 × *g* for 2 min, the sediment was discarded, and the supernatant was recentrifuged at 1000 × *g* for 15 min to obtain whole chloroplasts. The chloroplasts were resuspended in the same phosphate buffer and were stored in the dark at 0° until used. The change, caused by light, of the turbidity at 750 mμ of the chloroplasts prepared as described above was practically the same as that reported by ITOH *et al.*[2]. The turbidity of a chloroplast suspension in 0.04 M phosphate buffer increased from 0.554 to 0.576 on illumination. This turbidity increase was completely reversible.

Reaction mixtures

An aliquot of a chloroplast suspension was divided into two parts. One part (2 ml) was pre-illuminated for 10 min with the light from a white fluorescent lamp. The illuminated suspension was mixed with 5 ml of 1 M bicarbonate–NaOH buffer at pH 8.8, and 1 ml of a diazonium-1,2,4-triazole solution was added to the mixture. To the reaction mixture kept standing for 20 min in the light at room temperature ($15 \pm 5°$) 1 ml of a 1% sodium azide solution was added. The addition of sodium azide completely stopped the reaction as described by TAKENAKA *et al.*[14]. The other part was kept in the dark for 10 min in the same phosphate buffer and was similarly treated with diazonium-1,2,4-triazole in the dark. These chloroplast suspensions treated with diazonium-1,2,4-triazole, one in the light and the other in the dark, were solubilized with a nonionic detergent, Emulgen-810 (polyoxyethylene octyl phenol ether), and were subjected to photometric measurements after 30 min of incubation with the detergent. The procedure after solubilization was carried out under dim light to minimize the effect of bleaching of chlorophylls. Other modification reagents were applied in the same manner, although the conditions in the treatment were different for each reagent as described later.

Spectroscopic measurements

Absorption spectra of chloroplast suspensions (in terms of the semi-integral attenuance[15], ${}_pE_t$) free from the effect of forward scattering were measured by placing the sample close to the large photocathode of the end-on type of a Shimadzu Multipurpose recording spectrophotometer model MPS-50, using 1.0-cm cells. Both light and dark samples of diazonium-1,2,4-triazole-treated chloroplasts after solubilization with Emulgen were slightly turbid. These samples were subjected to turbidimetry. The turbidity (in terms of rectilinear attenuance[15], ${}_pE_p$) was measured by placing the sample with a narrow slit at a remote position, 12 cm, from the photocathode[3]. The suspensions for the absorption spectrophotometry and turbidimetry contained chloroplasts equivalent to 13 μg chlorophylls per ml.

RESULTS

Absorption spectra of chloroplasts treated with diazonium-1,2,4-triazole in the dark and in the light

Diazonium compounds react with histidine and tyrosine side chains and with amino groups of lysine or at peptide termini to form monoazo and bisazo derivatives. The reactions of diazonium-1,2,4-triazole with proteins were examined as a preliminary experiment because this diazonium compound has not been employed for protein modification. The spectra of 1 μM of lysozyme and 3 μM of albumin treated with excess diazonium-1,2,4-triazole (8.5 mM) showed a strong band of monoazo derivatives of these amino acid side chains around 360 mμ and a weak band of their bisazo derivatives at 480 mμ. These wavelengths agree with those found with other diazonium reagents[10,16]. In the measurements of these absorption spectra, a solution of diazonium-1,2,4-triazole at the same concentration without proteins was used as the reference. The reaction was completed within 20 min at room temperature, and illumination of the sample with a white fluorescent lamp did not affect these absorption spectra.

A chloroplast suspension preincubated in the dark or in the light was treated with 8.5 mM diazonium-1,2,4-triazole for 20 min in the dark (dark sample) or in the light (light sample), respectively. Curve A in Fig. 1, which is the spectrum of chloroplasts preincubated in the dark, changed to Curve B on the treatment with diazonium-1,2,4-triazole in the dark. The absorption below 400 mμ was intensified remarkably by this treatment as exhibited more distinctly in the difference spectrum (Curve C) of Curve B *minus* Curve A. A prominent increase in absorbance below 400 mμ indicates that diazonium-1,2,4-triazole reacts with chloroplast proteins. A weak band at 520 mμ may be due to absorption by the bisazo derivatives of the side chains of proteins. Difference minima located at 440, 490 and 685 mμ indicate the bleaching of chlorophylls and, probably, carotenoids. The difference spectrum similarly obtained in the light is shown by Curve D which does not much differ from Curve C obtained in the dark.

The absorbance changes of chloroplasts at a single wavelength of 360 mμ by the treatment with diazonium-1,2,4-triazole in the dark and in the light were observed as a function of diazonium-1,2,4-triazole concentration, and the results are shown by Curves A and B in Fig. 2, respectively. Both curves rise gradually and reach nearly the same level at 12.6 mM of diazonium-1,2,4-triazole. It was thus found that the absorbance difference, if any, between the light and dark samples is not great enough to evidence a change in the resistivity toward chemical modification by light. There was also no significant change at 520 mμ caused by light.

Turbidity of diazonium-1,2,4-triazole-treated chloroplasts after solubilization

A much greater effect of light was found when the samples were solubilized and measured by turbidimetry. Curves A and B in Fig. 3 are the spectra observed in terms of turbidity for the solubilized light and dark samples, respectively. While light and dark controls solubilized with Emulgen but not treated with diazonium-1,2,4-triazole showed practically no difference in turbidity between 350 and 750 mμ,

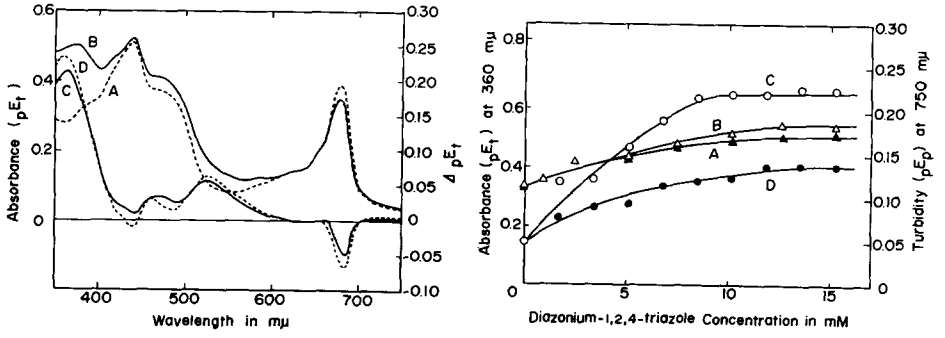

Fig. 1. Spectral changes of suspensions of chloroplasts on treatment with diazonium-1,2,4-triazole in the dark; Curves A and B, the spectra before and after treatment with 8.5 mM of diazonium-1,2,4-triazole, respectively. Curves C and D are difference spectra before and after treatment of chloroplasts with 8.5 mM of diazonium-1,2,4-triazole in the dark and in the light, respectively.

Fig. 2. The change of absorbance at 360 mμ observed for chloroplasts treated with various concentrations of diazonium-1,2,4-triazole in the dark (Curve A) and in the light (Curve B). Curves C and D show the turbidity change of the solubilized samples of chloroplasts treated with various concentrations of diazonium-1,2,4-triazole in the light and in the dark, respectively. The concentration of Emulgen was 0.1 %.

Curves A and B, measured for diazonium-1,2,4-triazole-treated and solubilized samples, showed an appreciable turbidity difference particularly in the spectral region of weak light absorption between 500 and 600 mμ and above 700 mμ; the turbidity of the light sample was appreciably higher than that of the dark sample. Turbidimetry in further experiments was, therefore, performed at 750 mμ where chloroplast pigments show little light absorption.

Curves C and D in Fig. 2 show the turbidity readings at 750 mμ of solubilized light and dark samples as a function of diazonium-1,2,4-triazole concentration. Whereas no difference was observed in turbidity between light and dark samples without diazonium-1,2,4-triazole, light and dark samples treated with diazonium-1,2,4-triazole above 8.5 mM showed a turbidity difference of 0.03. The slope of Curve C for light samples at zero diazonium-1,2,4-triazole concentration is much steeper than that of Curve D for dark samples. This difference in turbidity, $\Delta_p E_p$, between light and dark samples is taken to reflect a change by illumination of the resistivity of chloroplast proteins toward diazonium-1,2,4-triazole modification. Concentrated solutions of diazonium-1,2,4-triazole above 8.5 mM were vesicatory, so that further experiments were conducted with 8.5 mM diazonium-1,2,4-triazole. The difference in turbidity between the light and dark samples at this concentration of diazonium-1,2,4-triazole depended considerably on the sample chloroplasts. In general, the turbidity at 750 mμ of a solubilized light sample treated with 8.5 mM diazonium-1,2,4-triazole was 1.5–2.5-fold higher than that of the dark sample similarly treated.

Similar experiments were conducted with several other reagents; *p*-diazobenzenesulfonic acid, diazonium-1-H tetrazole, sodium 2,4,6-trinitrobenzenesulfonate and sodium β-naphtoquinone-4,6-disulfonate. The concentration of these reagents was 8.5 mM, the same concentration of diazonium-1,2,4-triazole in the above experiment. The increment, $\Delta_p E_p$, obtained with *p*-diazobenzenesulfonic acid as the reagent was 0.008 which was only 11 % of the increment, 0.075, obtained with diazonium-1,2,4-triazole (Table I). In the case of diazonium-1-H-tetrazole, the value of $\Delta_p E_p$ was as low as 8 % of the value obtained with diazonium-1,2,4-triazole. Practically no increment was observed between light and dark samples treated with sodium β-

TABLE I

RESISTIVITIES OF LIGHT AND DARK CHLOROPLASTS TOWARD REAGENTS AS MEASURED BY TURBIDIMETRY

Chloroplasts were treated with diazonium compounds in 0.625 M carbonate buffer (pH 8.8) for 20 min, with sodium β-naphtoquinone-4,6-disulfonate in 0.1 M phosphate buffer (pH 8.8) for 2 h and with sodium 2,4,6-trinitrobenzenesulfonate in 0.4 M carbonate buffer (pH 9.3) for 0.5 h.

Reagent	${}_pE_p$ *at 750 mμ*		$\Delta_p E_p$	
	Light	*Dark*		
Diazonium-1,2,4-triazole	0.140	0.065	0.075	(100 %)
p-Diazobenzenesulfonic acid	0.072	0.064	0.008	(11 %)
Diazonium-1-H-tetrazole	0.053	0.047	0.006	(8 %)
Sodium 2,4,6-trinitrobenzenesulfonate	0.050	0.048	0.002	(3 %)
Sodium β-naphtoquinone-4,6-disulfonate	0.044	0.044	0	(0 %)
Nontreated	0.033	0.033	0	(0 %)

naphtoquinone-4,6-disulfonate in 0.1 M phosphate buffer (pH 8.8) for 2 h or treated with sodium 2,4,6-trinitrobenzenesulfonate in 0.4 M bicarbonate buffer (pH 9.3) for 0.5 h. Diazonium-1,2,4-triazole was thus found to be the most suitable reagent for observing the turbidity change.

In the experiment of Fig. 4, the turbidity was measured as a function of Emulgen concentration for the DT-treated light and dark samples. As seen from Curve A showing a change in the turbidity of a light sample, the turbidity increases and attains a maximum value at 0.02 % of Emulgen. When Emulgen concentration was raised above 0.02 %, however, the turbidity dropped steeply and then gradually above 0.1 % to reach a lower constant value. Microscopic examination revealed that diazonium-1,2,4-triazole-treated chloroplasts underwent remarkable shrinkage on the treatment with Emulgen below 0.01% and were distintegrated into smaller particles at higher Emulgen concentrations. The suspension of chloroplasts treated with diazonium-1,2,4-triazole in the dark (Curve B in the same figure) shows an initial increase of turbidity below 0.01 % of Emulgen, the concentration being about half the concentration required for disintegration of chloroplasts treated with diazonium-1,2,4-triazole in the light. Above 0.01 %, the turbidity decreased steeply and approached a constant level at 0.05 %. The turbidity of nontreated chloroplasts dropped steeply below 0.05 % and was constant at higher concentrations as shown by Curve C in the same figure. It may be concluded from these results that the treatment of chloroplasts with diazonium-1,2,4-triazole results in a decrease of the solubility of chloroplasts proteins in aqueous Emulgen solution and that chloroplasts treated in the light are less soluble than those treated in the dark. In further experiments, the turbidity of light and dark samples was measured after solubilization with 0.1% Emulgen. The solubilization of diazonium-1,2,4-triazole-treated chloroplasts at such an excess concentration of Emulgen was completed within 10 min, and the turbidity reading stayed constant for at least 60 min. The reading in the experiments described below was therefore made after 30 min of incubation.

Chloroplasts were more soluble with sodium dodecyl sulfate than with Emulgen (Table II). However, the turbidity difference was smaller with sodium dodecyl sulfate; the increment, $\Delta_p E_p$, obtained with sodium dodecyl sulfate in an experiment was 37 % of that obtained with Emulgen.

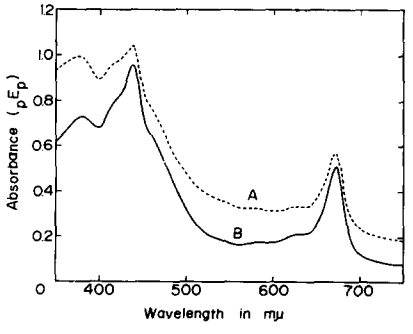

Fig. 3. Spectra in terms of turbidity of the solubilized samples of chloroplasts treated with 8.5 mM of diazonium-1,2,4-triazole in the light (Curve A) and in the dark (Curve B).

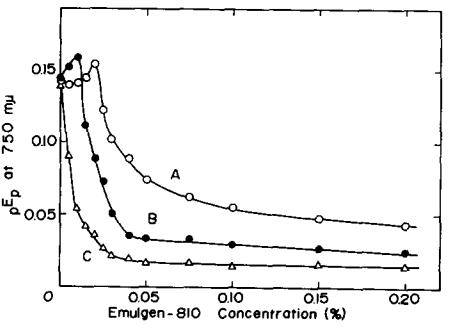

Fig. 4. Effect of Emulgen concentration on the turbidity of chloroplasts treated with 8.5 mM of diazonium-1,2,4-triazole; Curves A and B, treated with diazonium-1,2,4-triazole in the light and in the dark, respectively; Curve C, chloroplasts without diazonium-1,2,4-triazole.

The effects of phenazine methosulfate, ATP and inhibitors

Pentachlorophenol[17], 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU)[18], 2-methylthio-4,6-bis-isopropylamino-*s*-triazine(prometryn)[19] and *o*-phenanthroline[19] are known to inhibit the Hill reaction. Chloroplasts with phosphate were incubated with one of these inhibitors for 10 min in the dark and then the increment, $\Delta_p E_p$, due to illumination was examined (Table III). In the presence of 0.5 mM CMU the turbidity of the solubilized sample of chloroplasts treated with diazonium-1,2,4-triazole in the light was 0.076, which was remarkably lower than the reading, 0.102, obtained by the same treatment without the inhibitor. On the other hand, the turbidity of the solubilized sample of chloroplasts treated with diazonium-1,2,4-triazole in the dark in the presence of CMU was practically the same as that obtained by the same treatment with diazonium-1,2,4-triazole without the inhibitor. The value of $\Delta_p E_p$ in the presence of CMU was 0.009 which was 28% of the control reading without the inhibitor. Similar results were obtained for samples preincubated with 0.3 mM pentachlorophenol or 0.75 mM prometryn; the light-induced increase of turbidity was suppressed by 78 and 62% in the presence of pentachlorophenol and prometryn, respectively. By contrast, the turbidity of solubilized samples of chloroplasts treated with diazonium-1,2,4-triazole in the light in the presence of

TABLE II

THE EFFECT OF DETERGENTS ON CHLOROPLASTS TREATED WITH DIAZONIUM-1,2,4-TRIAZOLE IN THE LIGHT AND IN THE DARK AS MEASURED BY TURBIDIMETRY

The concentration of detergents was 0.1%.

Detergent	$_pE_p$ *at 750 mμ*		$\Delta_p E_p$
	Light	*Dark*	
Emulgen	0.100	0.048	0.052 (100%)
Sodium dodecyl sulfate	0.072	0.053	0.019 (37%)

TABLE III

THE EFFECTS OF PMS, ATP AND INHIBITORS ON THE TURBIDITY READINGS OF SOLUBILIZED LIGHT AND DARK CHLOROPLASTS TREATED WITH DIAZONIUM-1,2,4-TRIAZOLE

Chloroplasts were incubated with the inhibitors or PMS for 10 min in the dark before the treatment with diazonium-1,2,4-triazole. ATP was added to chloroplasts just before the treatment.

Compound	$_pE_p$ *at 750 mμ*		$\Delta_p E_p$
	Light	*Dark*	
Control (diazonium-1,2,4-triazole-treated without inhibitor)	0.102	0.070	0.032 (100%)
0.5 mM CMU	0.076	0.067	0.009 (28%)
0.3 mM pentachlorophenol	0.087	0.080	0.007 (22%)
0.75 mM prometryn	0.085	0.073	0.012 (38%)
0.25 mM *o*-phenanthroline	0.114	0.085	0.029 (91%)
0.04 mM PMS	0.106	0.068	0.038 (119%)
3.75 mM ATP	0.113	0.081	0.032 (100%)

0.25 mM *o*-phenanthroline was higher than that obtained without the inhibitor, and the value of $\Delta_p E_p$ was as high as 91% of the control reading. Experiments to examine the effect of NH_4Cl, an uncoupler of photophosphorylation, were unsuccessful because NH_4Cl reacted with diazonium-1,2,4-triazole.

A similar experiment was conducted with phenazine methosulfate (PMS), known as an electron carrier. The turbidity of a solubilized sample of chloroplasts treated with diazonium-1,2,4-triazole in the light in the presence of 0.04 mM PMS was 0.106 which was higher than the reading obtained without PMS; the value of $\Delta_p E_p$ with PMS was 0.038 which is 19% higher than that obtained for the control. The addition of ATP caused a much greater increase of turbidity both in the light and in the dark; in the presence of 3.75 mM ATP, the turbidity readings of the solubilized samples of chloroplasts treated with diazonium-1,2,4-triazole in the light and in the dark were 0.113 and 0.081, respectively. Light and dark chloroplasts stored at 0° for 2 days or heated at 50° for 10 min showed no difference in turbidity after treatment with diazonium-1,2,4-triazole and solubilization. The chloroplasts pretreated for 3 min with a detergent such as sodium dodecyl sulfate (0.1 %) or Emulgen (0.1 %) also showed no increase of turbidity by illumination.

DISCUSSION

It was demonstrated in the present study that the turbidity of a solubilized sample of chloroplasts treated with diazonium-1,2,4-triazole in the light was about 2-fold higher than that of an identical sample treated with diazonium-1,2,4-triazole in the dark. This increase in turbidity due to light may be interpreted as being due to a change in the resistivity toward chemical modification of chloroplast proteins or a change in the number of accessible binding sites for this reagent as a consequence of a change of chloroplast configuration.

The light-induced turbidity increase was inhibited to the same extent as the Hill reaction was inhibited by inhibitors such as CMU, pentachlorophenol and prometryn. ITOH *et al.*[2] demonstrated that the addition of ATP lowers, instantaneously, the volume of chloroplasts both in the light and in the dark. A similar effect of ATP on the turbidity increase was observed in the present experiments (Table III). It may therefore be inferred that ATP as well as light accomplishes this *via* an indirect process, affecting electron flows.

BERG[20] successfully employed *p*-diazobenzenesulfonic acid for radioactive labelling of outer components of the human erythrocyte membranes. Diazonium-1,2,4-triazole gave a greater difference of turbidity than *p*-diazobenzenesulfonic acid in the present experiments for chloroplast proteins. The success of the present study in observing a light-induced change of chloroplast proteins in their resistivity toward modification may be due to the following characteristics of diazonium-1,2,4-triazole. (a) The reagent possesses a high reactivity with proteins at room temperature and weakly alkaline pH and does not afford colored by-products during its reaction with proteins. (b) The solubility of chloroplast proteins in a nonionic detergent solution is lowered considerably by the treatment with diazonium-1,2,4-triazole. (c) A triazole ring in the structure of diazonium-1,2,4-triazole may possess a specific affinity for chloroplast membranes because the Hill reaction of chloroplasts was found to be

inhibited appreciably by 3-amino-1,2,4-triazole in an experiment in our laboratory[21]; the degree of inhibition was 60 % at 8.5 mM of this triazole reagent.

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