RIBULOSE DIPHOSPHATE CARBOXYLASE I. A FACTOR INVOLVED IN LIGHT ACTIVATION OF THE ENZYME*

by

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SUMMARY

The enzyme ribulose diphosphate carboxylase (RudPCase) has been shown to be present in high concentrations in chloroplasts (Lyttleton and Tso, 1958); however, the measured turnover of the isolated enzyme is so low that the exact role of this enzyme in CO_2 fixation has been questioned (Trown, 1965; Gibbs *et al.*, 1967). In addition the levels of CO_2 required for efficient functioning of purified RudPCase are so high relative to the concentration of CO_2 in the atmosphere that calculations by various workers have concluded that the enzyme could not support the known CO_2 fixation rate in intact tissue. As a result, extensive investigations have been carried out to determine means of preserving or restoring activity to the isolated enzyme. This communication describes the isolation of a small factor both from tomato leaves and from isolated chloroplasts which increases the enzyme activity of ribulose diphosphate carboxylase both in crude extracts and in the isolated enzyme preparations. The factor has a chromophoric group with absorption maxiumum at 325 mμ. Moreover, the activation of RudPCase is light dependent with a maximum in the action spectrum also at 325 mμ.

MATERIALS AND METHODS

Ribulose diphosphate carboxylase activity measurements were carried out by observing the rate of $^{14}CO_2$ fixation into acid stable products as described by Willemot and Criddle (1970). Enzyme extracts were prepared by grinding 1 gm leaf tissue with 2 ml HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, pH 8.0. The suspension was centrifuged 10 min at 48,000 x g to remove debris and then again at 105,000 x g for 1 hour. This supernatant fraction was then used for assays. The tomato enzyme was purified further as described by Andersen *et al.* (1970). Enzyme assays were carried out in 1 ml quartz cuvettes. Incubations for measurement of light dependent activation were carried out in the sample compartment of a Gilford-modified Beckman DU

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spectrophotometer. Generally 325 mμ light and a 0.1 mm slit width were employed for assay. For measurement of the reaction in absence of light, the cuvettes were covered with dark brown glass bottles. The action spectrum for activation with Light Activating Factor (LAF) was also run in a Beckman DU spectrophotometer. While the absolute light intensity utilized in such measurements is unknown, the intensity over the entire spectral range employed was maintained essentially constant by adjustments of the slit width at each wave length employed to compensate both for spectral intensity and spectral response differences of the light source and photocell employed.

Chloroplasts were prepared from tomato leaves according to the procedures of Stumpf (1970) and isolation of LAF carried out as with the whole leaf extract.

Light activating factor was prepared from tomato leaf tissue (except where noted otherwise) by grinding 1 gm of leaf tissue with 2 ml cold absolute ethanol using a mortar and pestle. The resultant suspension was then clarified by centrifugation for 10 min at 48,000 x g. The alcoholic extract was evaporated to dryness and the residue dissolved in 0.1 M HEPES buffer containing 1 mM dithioerythritol, 1 mM EDTA, 250 μM $MgCl_2$ and 250 μM $NaHCO_3$ at pH 8.0. The insoluble material at this step was again removed by centrifugation at 48,000 x g for 10 min to yield a green pellet and a clear yellow supernatant fraction containing LAF. Further fractionation using ammonium sulfate precipitation was very dependent upon concentration of the LAF but precipitation could be initiated by 10%-20% ammonium sulfate. The precipitate was then collected by centrifugation and dissolved in the HEPES buffer described above. No differences in the light activation and activity stimulation properties of the extracts before and after ammonium sulfate fractionation were noted and therefore LAF prepared as above through the step of solubilization in buffer and centrifugation was used in many of the studies described.

Disc gel electrophoresis was run using the Ornstein-Davis buffer system (Davis, 1964). Absorption spectra were recorded with a Cary model 14 spectro-

photometer. Fluorescence measurements employed a Farrand recording spectrophotofluorometer. Protein was determined by the procedure of Lowry *et al.* (1951). Enzyme substrates and assay conditions were as described by Willemot and Criddle (1969).

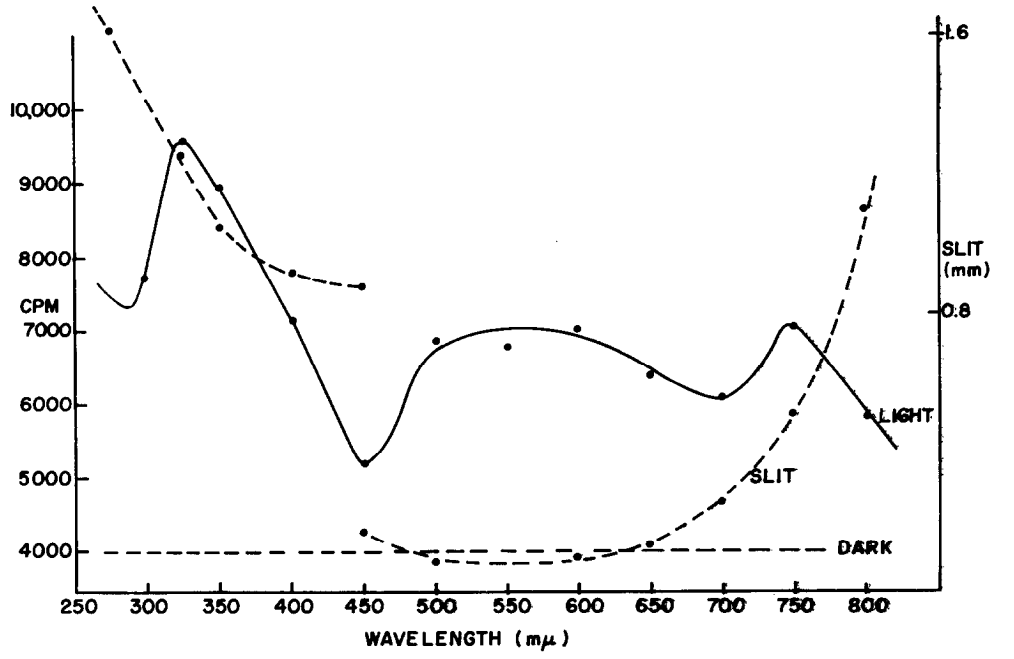
Table I. % Light Activation of Ribulosediphosphate Carboxylase

Enzyme Source	% Activation	% Activation with Added Pigment Fraction*
Photosynthetic Bacteria		
Rhodospirillum Rubrum	100	
Marine Algae		
Ilea	-100	
Iridophycus	100	
Postelsia	73	
Lycophyta		
Selaginella	21	125
Angiosperm		
Tobacco	36	47
Tomato	100	100
Spinach	50	
Wheat	25	28
Barley	13	36
Sugar Cane	79	100
Maize	-	63

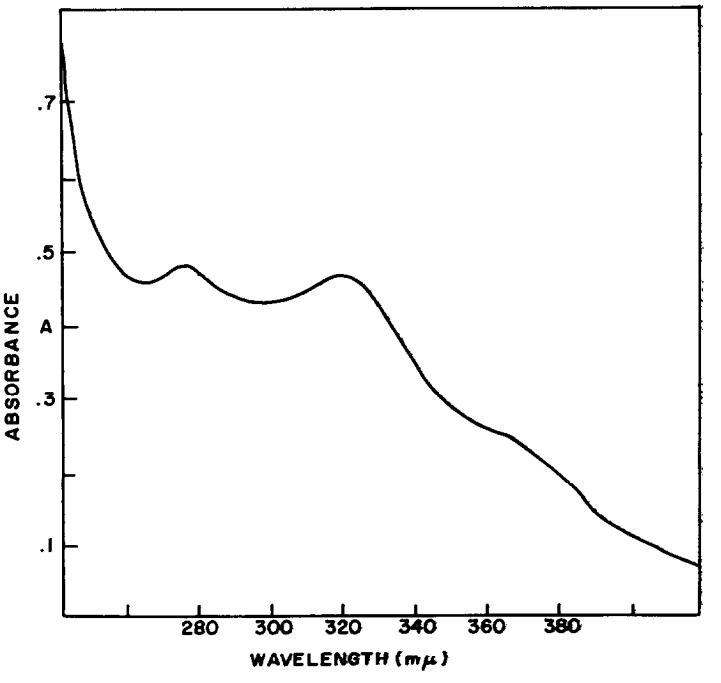
*Pigment fraction was HEPES soluble material from evaporated alcohol extract of the respective tissue sources.

RESULTS

Light activation of RudPCase activity has been observed in the crude extracts from many plant sources as indicated in Table I. In many cases this activation could be further enhanced by the addition of a preparation of LAF isolated from the respective plant sources. The maxiumum activation in such crude extracts is variable depending on plant used and generally does not exceed 100%. Variation in the absolute amount of activation may be greatly dependent upon growth conditions since major variations are observed upon repeated extraction of leaves of a given tomato plant type. Activation with the tomato plant may vary from a very low level of increase up to as much as 270% for a purified enzyme preparation.

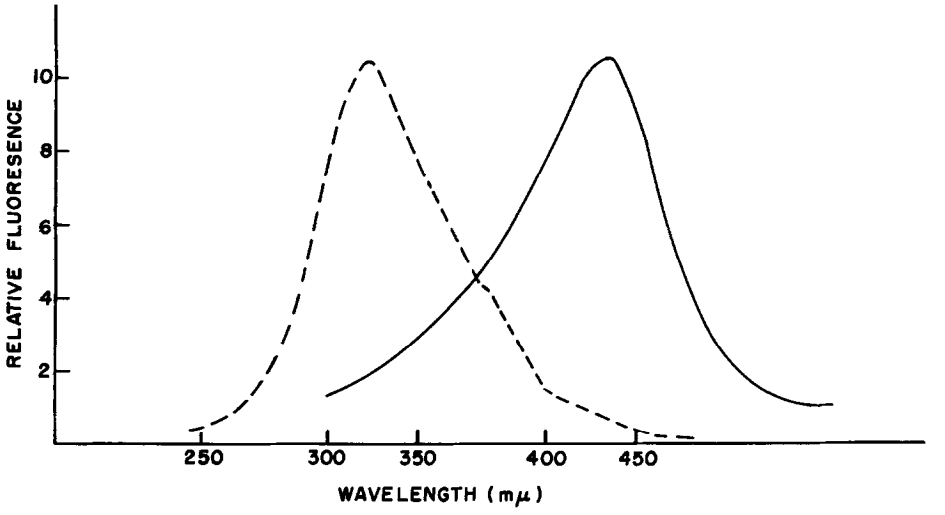


1. Action spectrum for light activation of tomato ribulose diphosphate carboxylase in pH 8.0 HEPES buffer. The solid line represents measured activity at the wavelengths tested. The lower dotted curve shows the base level of dark activity. The slit width utilized in the Beckman DU-spectrophotometer to maintain constant illumination energy at all wavelengths tested is shown by the dashed line.



2. Absorption spectrum of LAF in pH 8.0 HEPES buffer.

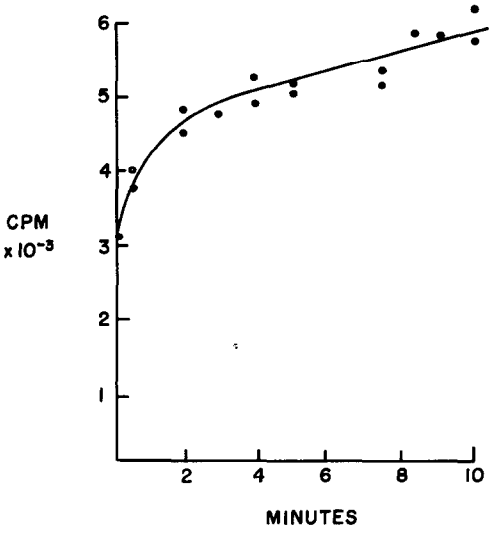
The action spectrum for light activation of RudPCase (Fig. 1) shows a maximum at 325 mμ with secondary shoulders near 550 and 750 mμ. The absorption spectrum of a purified preparation of the light activating factor in Fig. 2 shows a major absorption at 270 mμ and in addition a longer wavelength maximum at 325 mμ corresponding to that noted for activation. The light activating factor preparations obtained as outlined are strongly fluorescent showing three distinct fluorescence maxima. The first of these is the typical protein fluorescence with excitation at 280 mμ and emission maxiumum at 340 mμ.



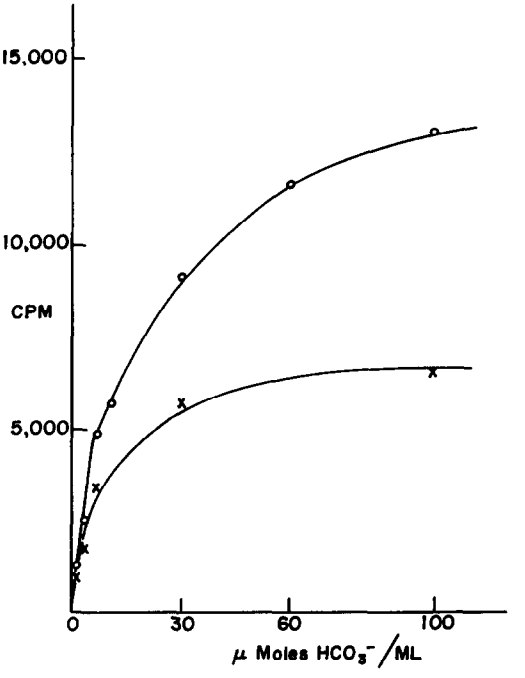
3. Activation and fluorescence spectra of LAF. The solid curve corresponds to fluorescence following activation at 325 mμ and the dashed curve to the activation spectrum obtained by observing fluorescence at 440 mμ.

Exciting at 325 mμ yields a fluorescent maxiumum at 440 mμ (Fig. 3). Excitation with 360 mμ light gives a secondary lower maximum at 390 mμ. In the pH range less than pH 7, the latter of these fluorescence maxima disappears independently of any change in the 440 mμ fluorescence and thus suggests the presence of at least two absorbing species in our LAF preparations. Young plants grown in the spring of the year lack the 360 mμ absorbing component.

The light activation of RudPCase shows a time dependent light saturation. When the assay solution is illuminated with 325 mμ light for varying initial portions of the 10 minute assay, an initial rapid light activation is noted



4. Time dependence of light activation of ribulose diphosphate carboxylase. Samples were mixed and placed in the light (325 mμ) for the varying times indicated and then removed to the dark for the balance of the 10 minute incubation period.



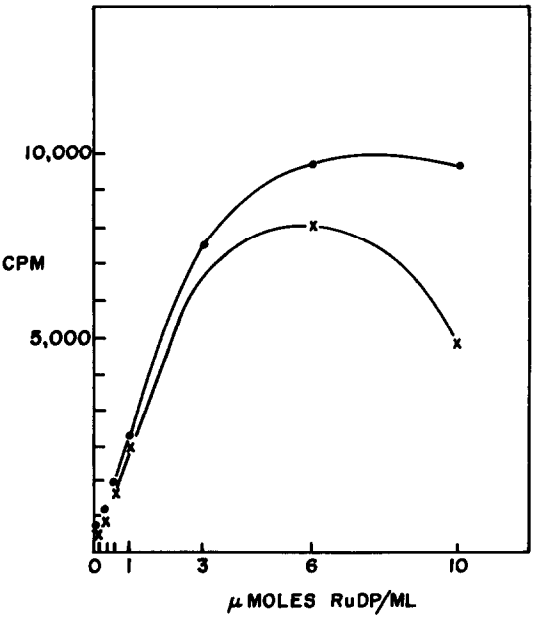
5. Effect of light and dark incubation conditions on the activity of RudPCase as a function of HCO_3^- concentration. Top curve, light; bottom curve, dark.

over the first 3 minutes of illumination followed by a near linear increase over the remainder of the incubation period as shown in Fig. 4.

The purified LAF preparations show a single rapidly migrating protein band using disc gel electrophoresis. Since at low gel concentrations this band migrates with the tracking dye front, it is necessary to employ acrylamide gel concentrations greater than 14% in order to achieve appreciable sieving and retardation of the protein. Based on this mobility dependence on gel concentration the molecular weight of the protein component is suggested to be in the range of 4,000 to 8,000 g/M.

The ability of LAF to activate the carboxylation reaction is stable to heating at 100° for 2 minutes.

The effects of light and LAF on the kinetic dependence of RudPCase activity on substrate concentrations are illustrated in Figs. 5 and 6. While the form of the curve of CO_2 dependence is not altered appreciably by LAF, it may be readily seen that efficient utilization of CO_2 is greatly enhanced. While 100 mM HCO_3^-



6. Effect of light and dark incubation conditions on the activity of RudPCase as a function of ribulose diphosphate concentration. Top curve, light; bottom curve, dark.

is generally required for maximal enzyme activity, light activation allows this same activity with only 1/10 the bicarbonate concentration. The shape of the kinetic curve obtained by varying ribulose diphosphate as well as the maximum rate is altered by the light dependent activation.

DISCUSSION

Light activation of the RudPCase reaction has been noted for a wide variety of plant sources and a photosynthetic bacterium. The activation is mediated by a small pigment containing protein fraction which can be readily isolated from plant extracts or from chloroplasts. In crude extracts, 100% light activation of RudPCase may be noted. With isolated and purified enzyme, the effect is much greater. The higher the degree of purification of RudPCase from tomato plants, the more dependent the enzyme becomes on the addition of LAF for activity and the light dependent activation.

Plants differ greatly in their ability to retain bound LAF during isolation. The tomato enzyme, and particularly some mutants thereof, can be readily isolated in a form lacking this factor. In this form there is no light dependence and only a very low activity. Readdition of LAF restores both light dependence and high specific activity. Mutant tomato plants lacking appreciable light activation in the crude extracts have also been identified (Andersen *et al.* 1970).

The existence of LAF and its observed effect on the RudPCase reaction may help to explain several of the problems associated with quantitation of enzyme activity relative to known rates of CO_2 incorporation. Such a factor could account for the *in vivo* blue light effect on CO_2 fixation first noted by Warburg *et al.* (1954). Also LAF could be involved in the observed increase in rate of CO_2 fixation in chloroplasts with increasing white light intensity noted by Turner *et al.* (1962). At present it is not clear whether enzymes from all plant sources have a similar dependence on such a small factor and whether light activation is important in all such cases. The general phenomenon does seem to be widespread but not universal under our assay conditions.

The mechanism by which LAF influences enzyme activity in the various

plants is not yet known. While protein has been universally found associated with the 325 mμ absorbing pigment, the required role of this protein has not been established. The variability observed among plants may be due to differing ratios of LAF and enzyme or by the presence of other factors affecting activity as well as by specific differences in RudPCase from individual plants. While an activation of enzyme activity is noted, this may equally well be considered as a reversal of inhibition by a second factor or reversal of an inactivating structural change. A further consideration of the structure and mechanism of action of LAF is under way to answer these questions.

The general nature and properties of the activating factor are very reminiscent of the protein factor first described by Saltman and Gee (1966) which was involved in activation of aerobic photophosphorylation and also of a similar factor with this role recently described by Wu and Myers (1969). Absorption and fluorescent spectra while slightly different in peak positions are similar. Stability to heat denaturation, acid stability and small size are also common features. It is possible that this type of molecule may play a general role in regulation of the entire photosynthetic process with a single activator species or a class of very similar components involved in several reaction sequences.

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