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ISOLATED FROM GLUTARALDEHYDE FIXED SPINACH LEAVES

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THE HILL REACTION OF CHLOROPLASTS ISOLATED FROM GLUTARALDEHYDE FIXED SPINACH LEAVES*

by

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In the course of studying effects of various hydrolytic enzymes on chloroplast fine structure, we found it useful to isolate chloroplasts from leaves previously fixed in neutral 6% glutaraldehyde.[1] Chloroplasts isolated in this way were not only morphologically indistinguishable from *in vivo* chloroplasts, as viewed by light microscopy or by electron microscopy on freeze-etched preparations, but also retained the optical rotatory dispersion and absorption spectra of unfixed chloroplasts. These initial observations indicated that the environment of chlorophyll was not greatly changed during glutaraldehyde fixation, and encouraged us to look for photochemical activity in these chloroplasts. In this paper we show that chloroplasts isolated from glutaraldehyde fixed leaves perform both the ferricyanide Hill reaction, as assayed manometrically by O_2 evolution, and indophenol reduction. The Hill reaction of these fixed chloroplasts is sensitive to reagents such as DCMU,** methylamine, and phosphorylation

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**Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ADP, adenosine diphosphate.

cofactors, and proceeds with an efficiency 25% that of unfixed chloroplasts. The Hill reaction capacity of the fixed chloroplasts is almost unchanged after storage periods of many weeks. These results indicate that quantum conversion and electron transport in photosynthesis are explainable in terms of a rigid protein framework with lipid dispersed through it. Conformational changes as such are apparently not necessary for quantum conversion, O_2 evolution and electron transport.

Materials and Methods.--The glutaraldehyde used for fixation was prepared by diluting the concentrated reagent with water to 6% (v/v), passing it through a Dowex-1 (Cl) column, and finally titrating it with KOH to pH 7.5.

Fixation: Spinach leaves were cut with a razor blade into 1-2 cm squares and placed in a beaker which contained an equal volume of 6% glutaraldehyde at room temperature. The beaker was then placed in a vacuum desiccator, pumped for about 2 minutes, and then returned to atmospheric pressure. This operation was repeated four times to completely fill the intercellular spaces of the leaf with fixative. The beaker containing the leaves and glutaraldehyde solution was then placed in the dark at room temperature for periods of time ranging from 20 minutes to 3 hours. Fixation times in excess of one hour yielded no further changes in the photochemical properties of the chloroplasts as indicated by retention of Hill reaction efficiency.

Chloroplast isolation: After fixation, the leaves were ground either with sand in a mortar or in the micro attachment for the Waring Blender operated at low speed. These operations were performed at room temperature without buffer, but with addition of a small amount

of water to the residual 6% glutaraldehyde. Grinding times from 2 to 10 minutes were adequate to disperse the fixed tissue. The slurry was then poured through 8 layers of cheesecloth and centrifuged at top speed (about 300 xg) in a clinical centrifuge for 2-3 minutes at room temperature. The precipitate contained beautifully preserved chloroplasts, nuclei and some mitochondria. This crude preparation was then washed 4 times in distilled water, which brought about no detectable morphological changes in the organelles (see Fig. 1). Washing was necessary to remove an unidentified reductant present in the homogenate. Otherwise the reductant reacted in the dark with the Hill oxidants. This washed precipitate was used for the experiments reported below. Although no effort was made to separate the various organelles, such separation should be easily achieved in a density gradient.

Unfixed chloroplasts for control experiments were isolated by homogenizing either in 0.5 M sucrose, 0.1 M (K) PO_4 pH 7.4, or in distilled water followed by fractional centrifugation.[2]

Reagents: Stock solutions of ferricyanide, ferrocyanide, and DCPIP (K & K Laboratories, Jamaica, N. Y.) were prepared on the day of the experiments. The DCPIP solution was prepared at pH 7.5, and the concentration determined using the extinction coefficients of Armstrong.[3] ADP was obtained from the Pabst Brewing Company, Milwaukee, Wisconsin, and DCMU from duPont de Nemours, Wilmington, Delaware. Methylamine hydrochloride was dissolved in distilled water and titrated to pH 7.4 with dilute NaOH.

Reaction mixtures: For the ferricyanide Hill reaction, the standard

reaction mixture (3 ml) contained the following in micromoles per ml: potassium ferricyanide 5, potassium ferrocyanide 5, methylamine 10, potassium phosphate pH 7.4 30, glutaraldehyde fixed chloroplasts containing 0.05-0.36 mg chlorophyll and, when added, DCMU, 1.9×10^{-2}. Sucrose (0.3 M) was included in some reaction mixtures. Its absence had no noticeable effect on oxygen evolution. The manometric determination of O_2 evolution was performed at 23° C. For the DCPIP Hill reaction, the standard reaction mixture contained in micromoles per ml the following: DCPIP 0.03, methylamine 10, potassium phosphate pH 7.4 35, and, when added, ADP 5, $MgCl_2$ 2.5, and sucrose, when present, 1000. This reaction was performed at room temperature. A Cary Model 14 spectrophotometer, modified according to Sauer and Biggins,[4] was used to determine quantum requirements for the DCPIP Hill reaction.

Results.--

Light microscopy of fixed chloroplasts: Chloroplasts and nuclei in the homogenate from glutaraldehyde fixed leaves showed no morphological response to changes in osmotic environment, but maintained their *in vivo* shapes, as shown in Fig. 1a. Fig. 1b is a photomicrograph of the same preparation after extraction of the pigments and lipids with 80% and 100% acetone. Except for color, the preparations in Figs. 1a and 1b are indistinguishable when observed by phase microscopy. The photomicrograph in Fig. 1c is an unfixed preparation of spinach chloroplasts after acetone extraction. The fact that the morphological integrity of the protein framework of the fixed membrane is preserved after lipid extraction opens the possibility of performing experiments on reconstitution of lipid and protein fractions and subsequent assay of photochemical properties.

Hill reaction of fixed chloroplasts: Fig. 2 presents the results of a mannometric assay of the ferricyanide Hill reaction by glutaraldehyde fixed chloroplasts. These data show that oxygen evolution by fixed chloroplasts is light dependent, and is inhibited by 1.9×10^{-5} M DCMU. Subsequent experiments have shown that the rate of O_2 evolution after the dark period is stimulated by addition of ferricyanide, even though only two thirds of the initial ferricyanide has been reduced (as calculated from O_2 evolved). Chlorophyll concentration was reduced in these experiments until saturation was achieved. At a light intensity of 3000 foot-candles, 0.17 mg chlorophyll/3ml of reaction mixture in standard 15 ml Warburg vessels appeared to yield a saturation rate. In this case the rate was 200 µl O_2/hr/mg chlorophyll. Though the saturation rate is low compared to attainable rates in unfixed chloroplasts,[5] the efficiency of quantum conversion at lower light intensities is surprisingly high. Efficiencies were determined for the DCPIP Hill reaction on both fixed and unfixed chloroplasts as a function of light intensity. These results are presented in Fig. 3. The zero intensity quantum requirement for DCPIP reduction by unfixed chloroplasts at 680 mµ is 2.4 einsteins/equivalent (a value in agreement with previous results), while that for fixed chloroplasts is 8.4. Chloroplasts isolated from leaves fixed only 20 minutes yielded a zero intensity quantum requirement of 3.4 for the Hill reaction with methylamine, while those from leaves fixed for 1 hour and 3 hours yielded quantum requirements of 8.7 and 10.0 respectively. DCPIP reduction by the fixed chloroplasts was completely inhibited by 1.9×10^{-5} M DCMU. Preliminary experiments have shown that the quantum requirement for DCPIP reduction

increased at wavelengths greater than 680 mμ. This red drop was also evident in fixed Chlorella cells (see Fig. 5).

The data in Fig. 3 also show that the intensity dependence of the quantum requirement for fixed chloroplasts is strongly affected by addition of methylamine or the cofactors of photosynthetic phosphorylation. We previously reported this effect,[2] first observed by Lumry et al.,[6] for unfixed chloroplasts in the ferricyanide Hill reaction and to a lesser extent in the indophenol Hill reaction. We interpreted this to mean that the light saturation of the Hill reaction begins at much higher intensity when phosphorylation either occurs rapidly or is uncoupled.[7-9] We supposed that the decreased effect of methylamine in the DCPIP Hill reaction was due to the uncoupling action of DCPIP itself at the concentration used. It is surprising that this effect remains in the fixed chloroplasts. Though we have not yet done experiments directly demonstrating phosphorylation by fixed chloroplasts, the above results do indicate that at least certain portions of the phosphorylation machinery remain coupled to electron transport.

The saturation rates of oxygen evolution in the ferricyanide Hill reaction and DCPIP reduction in the DCPIP Hill reaction appear, within experimental error, to be the same. The rates are equivalent to approximately 8 μM O_2/hr/mg chlorophyll. This relatively low saturation rate indicates that the fixation of the chloroplasts has imposed a rate limiting step on the Hill reaction which is not eliminated by methylamine. Efficiencies, however, are fairly high at lower light intensities where this step is no longer rate limiting as shown for the DCPIP Hill reaction.

We also investigated the effect of aging on the quantum requirement of the DCPIP Hill reaction. These results are shown in Fig. 4, and are plotted as quantum yields (the reciprocal of quantum requirements) rather than quantum requirements, to show the loss of efficiency with time. The experiments with unfixed chloroplasts, prepared in water or sucrose-phosphate, were discontinued after the first week, since these preparations became totally inactive and produced endogeneous reductants which produced large dark DCPIP reduction rates. The chloroplasts from leaves fixed for one hour or longer retained activity for very long periods of time even though stored in water at 4° C under air. A period of 24 days produced only a 30% decrease in quantum yield in chloroplasts from the one hour fixation. Our storage conditions for the experiments were not optimal and can easily be improved. For example, it is known that the fixation reaction itself is reversible, particularly under acidic conditions, and we stored the chloroplasts in complete absence of fixative. Also, the pigment absorption of the one hour sample decreased almost 50% during the 24 day storage under air. In the red region of the spectrum this loss occurred primarily as a decrease and broadening of the 678 mμ peak of chlorophyll. Storage under nitrogen in the presence of low concentrations of glutaraldehyde should produce preparations with still greater longevity.

Hill reaction in intact organisms: A suspension of the green alga, *Chlorella pyrenoidosa*, was fixed for 30 minutes in 6% glutaraldehyde. The cells were then washed 4 times with distilled water and were suspended in the DCPIP reaction mixture. The results of this experiment

are shown in Fig. 5. The cells become permeable to DCPIP during the fixation, by contrast with unfixed Chlorella cells, and yield results very similar to those found with chloroplasts from fixed leaves. There is a higher quantum requirement at 680 than at 668 mμ, indicating that the red drop phenomenon is still present. This method should have considerable use in studies of enhancement in cells in which the accessory pigment is easily lost during conventional chloroplast preparation procedures.

System 1 reactions: The reduced quinone-cytochrome c reaction mediated by system 1 was not detectable with fixed chloroplasts. We have not yet determined whether this lack of activity is due to a permeability barrier for cytochrome c, or whether system 1 is inactivated by fixation. Though no permeability barrier appears to exist for small molecules, such a barrier may still exist for molecules as large as cytochrome c.

CO_2 reduction: These chloroplasts do not incorporate $^{14}CO_2$ into organic compounds in either light or dark. We do not know the extent to which this inactivity results from denaturation of carbon cycle enzymes, loss of carbon cycle intermediates during distilled water washing of the chloroplasts, the inability of soluble cofactors to communicate among the reactive sites, or some combination of these effects.

Summary.--These experiments show that quantum conversion leading to O_2 evolution in the Hill reaction can occur in chloroplasts isolated from glutaraldehyde fixed leaves with about 25% the efficiency of unfixed chloroplasts. The Hill reaction seems normal in that it

DCMU inhibited, its rate is dependent on the presence of phosphorylation acceptors or uncouplers, and it exhibits a red drop in efficiency. The fixed chloroplasts do not change morphology upon acetone extraction, and therefore offer the possibility for performing lipid reconstitution experiments. Glutaraldehyde fixed Chlorella cells are also active in the DCPIP Hill reaction. These results indicate not only that quantum conversion and electron transport in photosynthesis is explainable in terms of a rigid protein frameword with lipid dispersed through it, but also that conformational changes as such are not necessary for quantum conversion leading to oxygen evolution and dye reduction.

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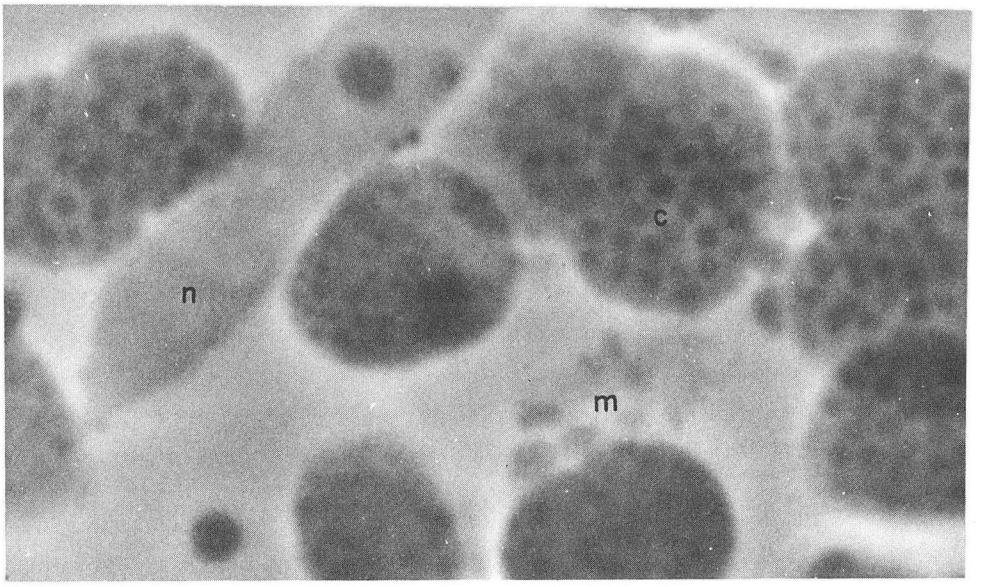

Fig. 1a. Chloroplasts isolated from glutaraldehyde fixed (3 hrs, 25°C) spinach leaves. Mitochondria (m) are still attached to some chloroplasts (c), and one nucleus (n) is present. Magnification 6200 x.

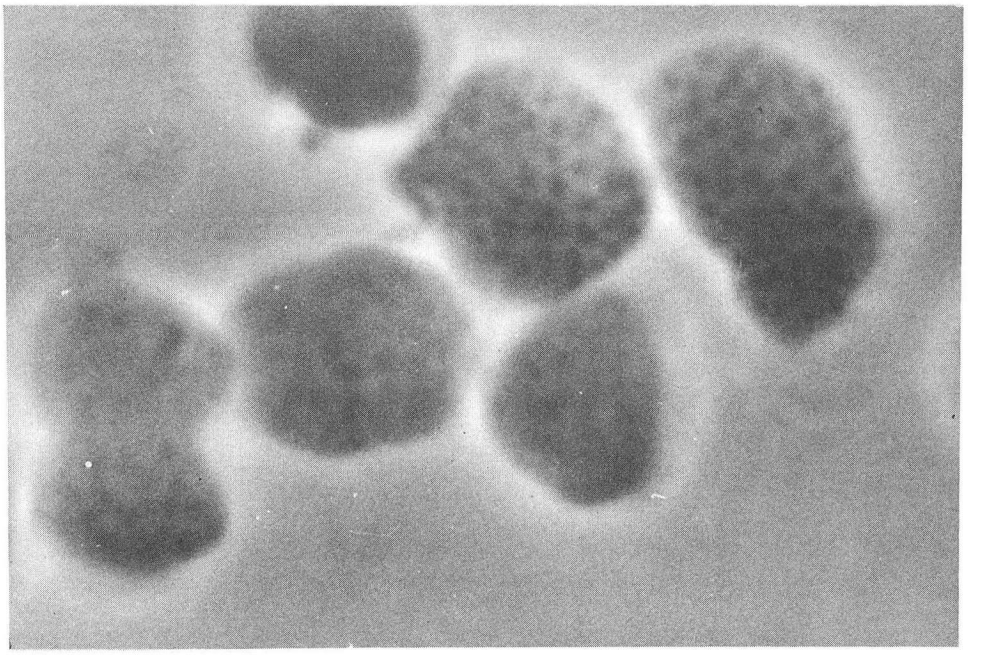

Fig. 1b. Preparation shown in Fig. 1a after extraction with 80% and 100% acetone. Magnification 6200 x.

Fig. 1c. Unfixed, sucrose phosphate isolated spinach chloroplasts after extraction with 80% and 100% acetone. Magnification 6200 x.

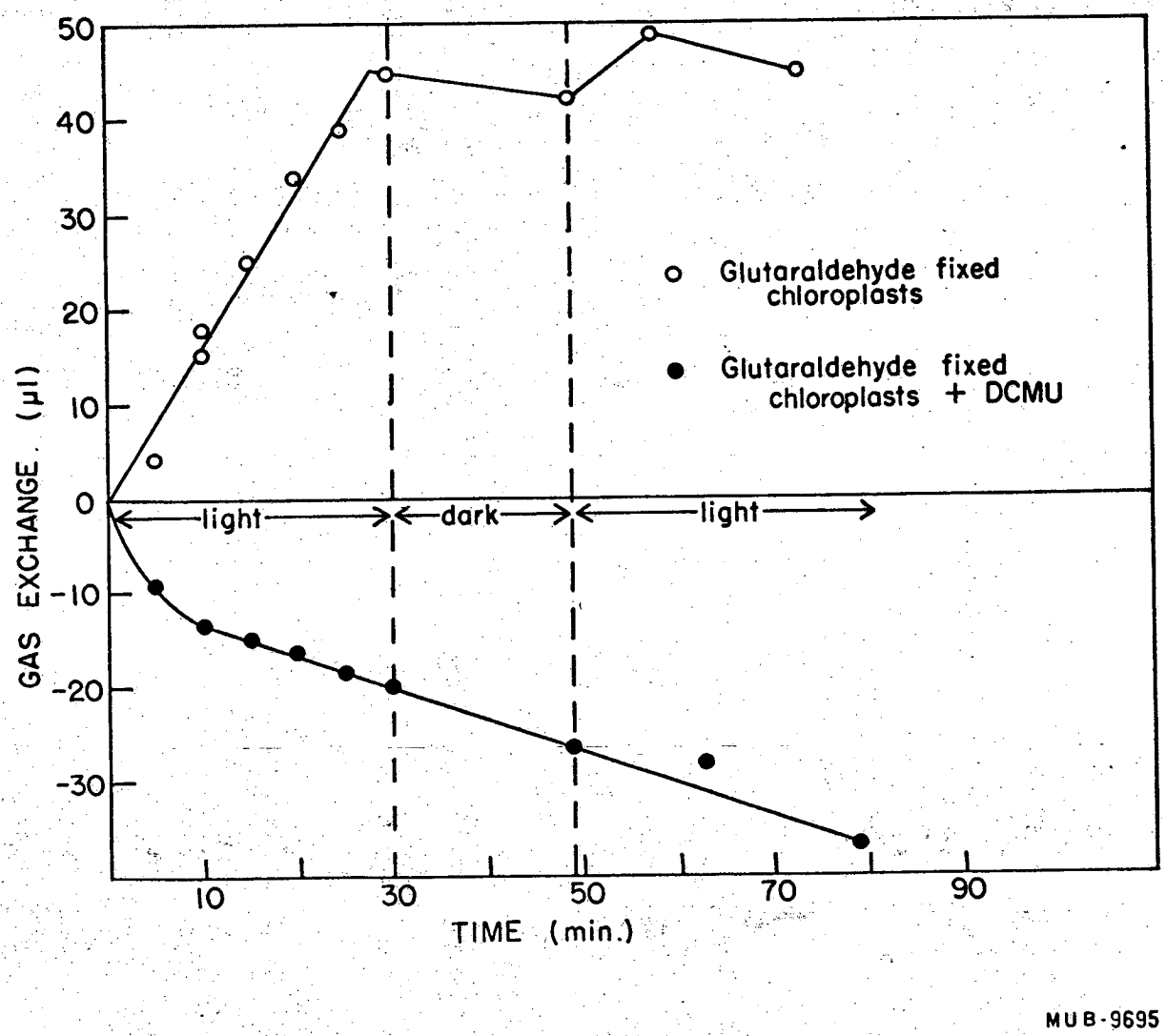

Fig. 2. Manometric determination of oxygen evolution by chloroplasts isolated from glutaraldehyde fixed leaves. Reaction mixture contained 1.09 mg chlorophyll in 3 ml. Other components given under Methods.

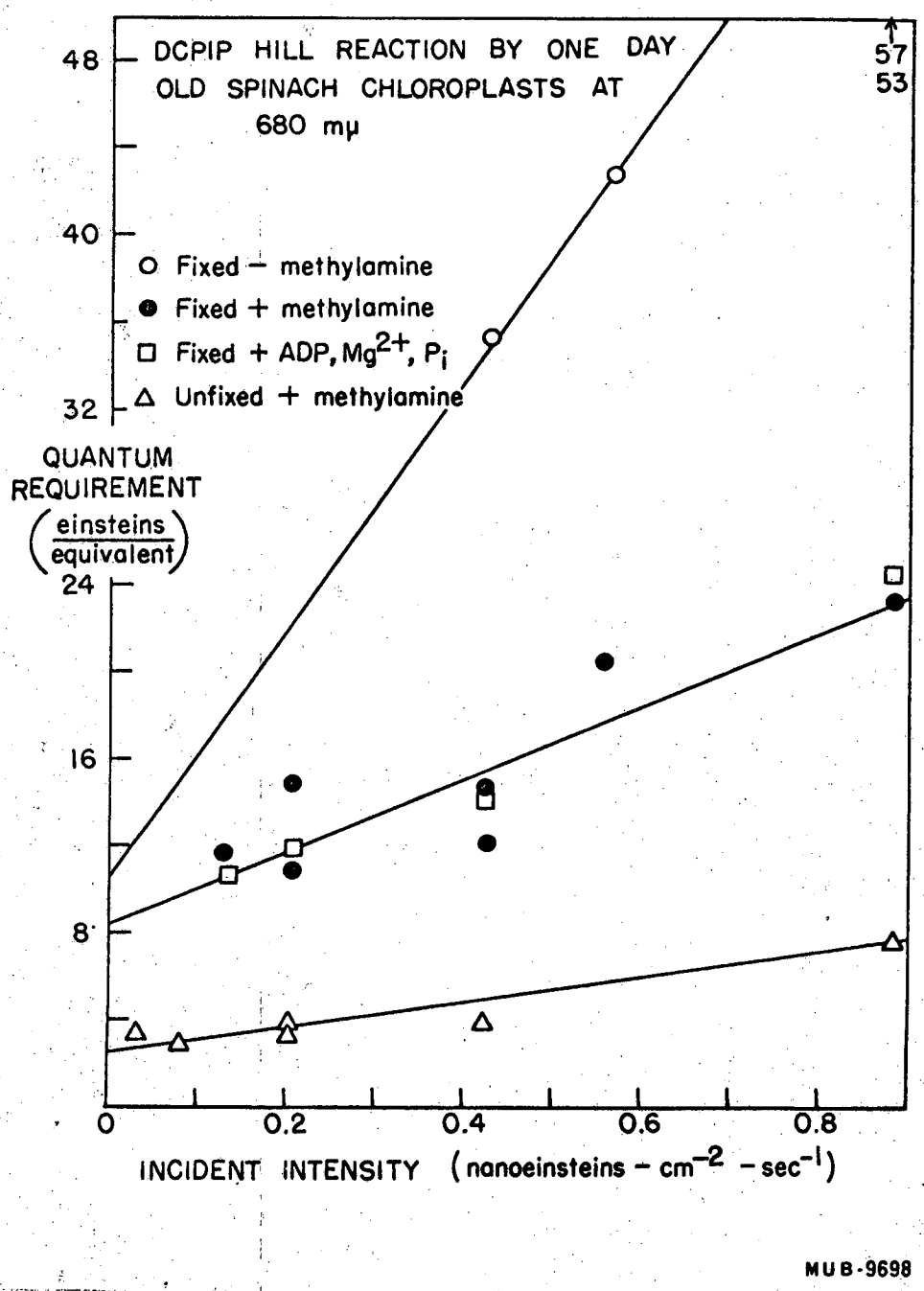

Fig. 3. Quantum requirements for DCPIP reduction by fixed and unfixed spinach chloroplasts.

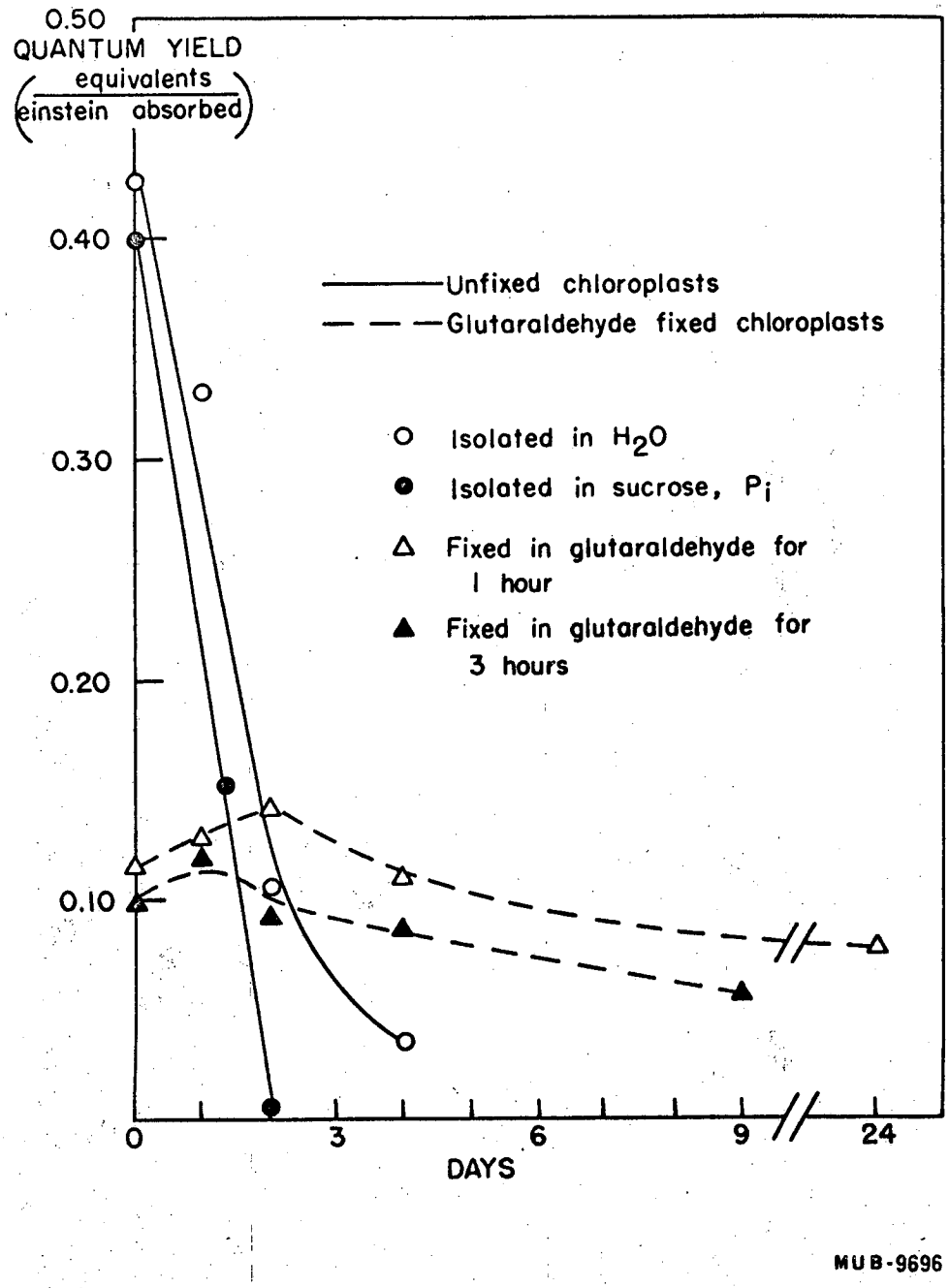

Fig. 4. The longevity of DCPIP reduction capacity of spinach chloroplasts isolated from fixed and unfixed leaves. Activity is reported as the extrapolated zero intensity quantum yields.

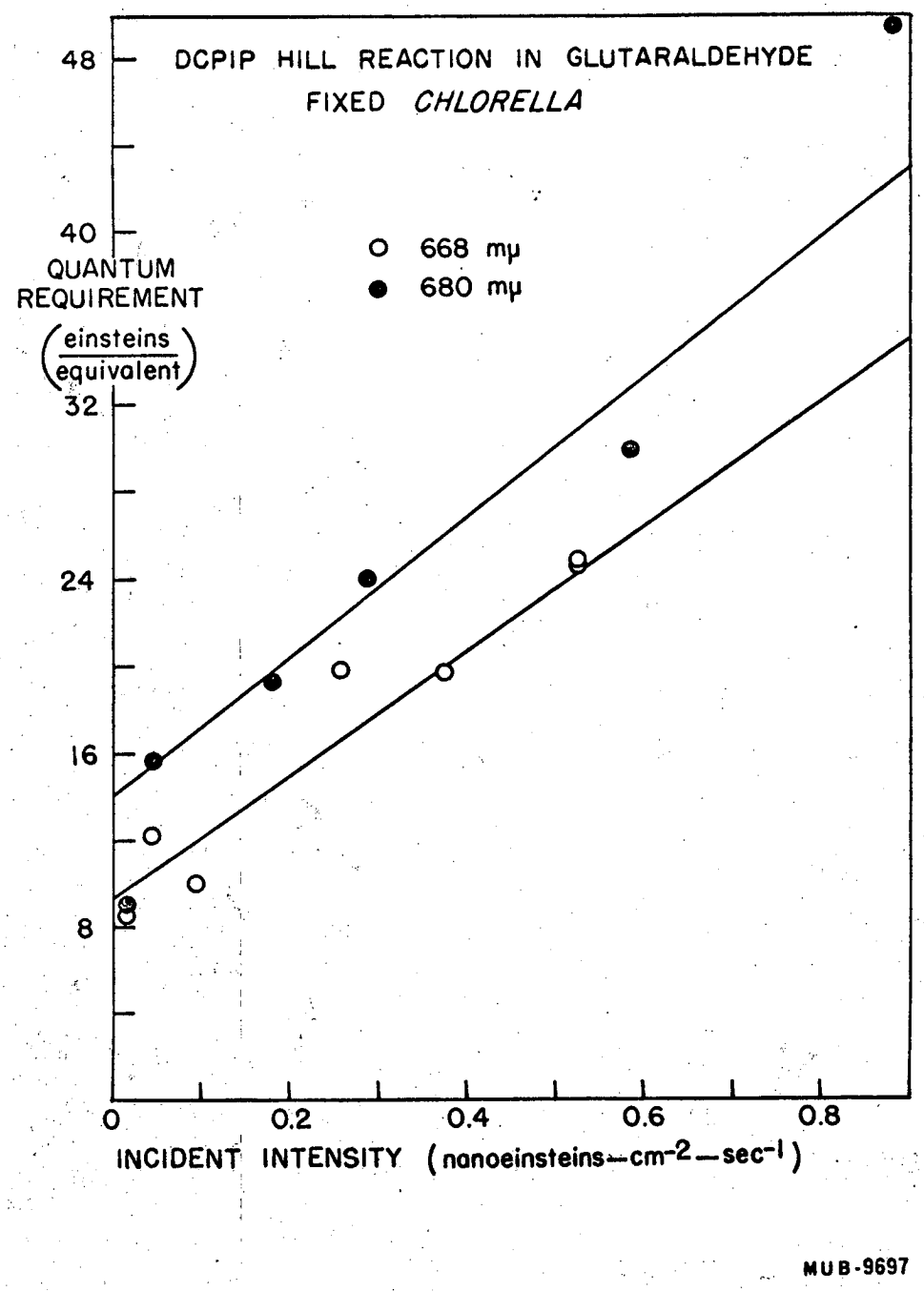

Fig. 5. Quantum requirements for DCPIP reduction by fixed Chlorella cells as a function of light intensity.

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