# Reports and Letters

## Reversible Bleaching of Chlorophyll in vivo

It has often been suggested (*1*) that in photosynthesis, chlorophyll undergoes a reversible change. It could be either (i) transformation into a "biradical," metastable state (such as an electronic triplet state, with both free valencies on the same atom, or a tautomeric state with the two valencies at different atoms); (ii) reduction, either to a semiquinone or to a valence-saturated leuco-compound; or (iii) oxidation, either to a radical or to a saturated product.

Transformation into the metastable state has been suggested as the first step in the internal conversion of excitation energy, which limits the yield of chlorophyll fluorescence to 25 to 35 percent *in vitro* (*2, 3*) and to 2 to 3 percent *in vivo* (*3*). According to Franck (*4*), photosynthesis probably occurs by reactions of metastable chlorophyll-*a* molecules. According to Livingston and Ryan (*5*), these molecules are coresponsible for changes in the absorption spectrum of illuminated chlorophyll solutions in the photostationary state; Livingston and Ryan (*5*) and Livingston, Porter, and Windsor (*6*), using condenser flashes with synchronized absorption measurements, found that during an intense flash up to 90 percent of chlorophyll in a $10^{-6}M$ solution can be present in the metastable state. Livingston and Ryan's (*5*) steady-state experiments indicated bleaching at 403 mμ, and enhanced absorption at 439.5 to 524.5 mμ, while their flash results showed bleaching at 468, 470.5, and 477.5 mμ and enhancement of color only at 524.5 mμ. However, according to the newer flash data of Livingston, Porter, and Windsor (*6*), analyzed by Livingston (*7*), enhancement extends to the range 450 to 560 mμ, with a sharp peak at 475 mμ and a shoulder at 520 mμ.

Evstigneev and Gavrilova (*8*) found that chlorophyll-*a*, photoreduced by phenylhydrazine in toluene, has absorption bands at 518 mμ and 585 mμ. Both bands were attributed to a semiquinone, the 518-mμ band to its ion, and the 585-mμ band to the nondissociated form. Krasnovsky (*9*) has suggested that chlorophyll participates in photosynthesis by reversible reduction to the semiquinone state.

Studies of reversible photobleaching of chlorophyll in $O_2$-free methanol (*2, 7, 10*) and of its reversible photooxidation by $Fe^{+++}$ in methanol (*11*) and by quinone in rigid solvents (*12*), as well as of the formation of the brown intermediate in the "phase test" (probably ionized enolchlorophyll, *13*), revealed an enhanced absorption by the unstable product in the region 450 to 550 mμ, but no sharp bands were detected, except in the last-named case, where a strong band at 524 and a weaker one at 683 mμ were noted.

It thus seems that, *in vitro,* reversibly reduced chlorophyll-*a* is characterized by bands at 525 mμ and 585 mμ and metastable chlorophyll-*a* by a band at 475 mμ; while reversible oxidation increases absorption in the same region, but apparently without producing a sharp new band. Enolization and ionization of chlorophyll-*a* leads to bands at 524 and 683 mμ.

Duysens (*14, 15*) noted that illuminated *Chlorella* cells showed, in addition to spectral changes attributable to the oxidation of a cytochrome (*14*), and perhaps also to the reduction of a pyridine nucleotide (*15*), a sharp new absorption band at 515 mμ and a somewhat smaller "negative" band (that is, selective decrease of absorption) at 478 mμ. He attributed the two changes to the transformation of an unidentified pigment, whose "dark" form absorbs at 478 mμ and whose "phototropic" form absorbs at 515 mμ. Witt (*16*) noted the 515-mμ band in plants exposed to an intense light flash. Duysens observed no change in the red region, thus apparently precluding the attribution of the effect at 515 mμ to chlorophyll (whose known phototropic forms are characterized by decreased absorption in the red band).

Using an apparatus similar in principle to that of Duysens (*17*) but with much stronger actinic light, we have been able to observe a decrease in absorption of illuminated *Chlorella* in the red. In our apparatus, the modulated photomultiplier output was amplified through three sharply tuned and six narrow band staggered stages; by means of a phase-inverting parallel twin-T tuned network, a considerable portion of the signal was negatively fed back from the fourth stage to the input. The ultrasharp tuning and increased feedback were necessitated by difficulty in discriminating between fluctuations in the fluorescence excited by the very intense actinic light and changes in the much weaker measuring light. After the ninth stage of amplification, the signal was rectified, compared, and, by means of a balanced-plate cathode follower, fed into a Brown Recorder (as in Duysens' instrument). Special checks convinced us that the observed difference spectrum was not significantly affected by changes in the fluorescence excited by the modulated photometric beam (which could possibly follow the exposure to the strong, nonmodulated actinic light).

*Chlorella* cells were grown in our laboratory, washed, suspended in carbonate, and refrigerated until they were used (*18*). The cells were used as taken from the refrigerator (optical density of suspension, 0.45, corrected for scattering at 680 and 740 mμ). The actinic light was furnished by a tungsten lamp 1000 w, GE 1000T20, 120 v); the entire side of the cuvette was uniformly illuminated. Before using a sample for systematic measurements (*19*), a check was made at several selected wavelengths to see whether the cells showed the normal response to illumination. The apparatus reproduced Duysens' earlier work with excellent agreement; in addition, it clearly showed absorption changes in the red.

A typical result is shown in Fig. 1. The optical density of illuminated cells is lower at 680 mμ by up to 0.25 percent. Although exact comparison with the increase at 515 mμ is not yet possible, because of the different excitation light that we had to use in the two regions, the two effects are of the same order of magnitude, thus permitting the assumption that they are both caused by a reversible change in chlorophyll-*a*. Spectroscopically this change is very similar to that observed by Krasnovsky (*9*), and by Evstigneev and Gavrilova (*8*) on reversible reduction of chlorophyll-*a* *in vitro* and by Weller (*13*) on ionization of chlorophyll enol.

The smaller changes farther in the red

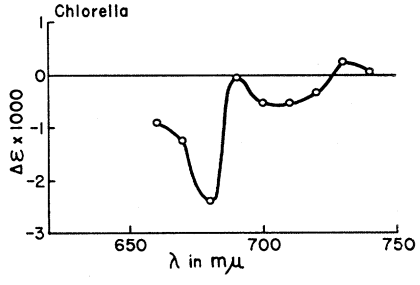


Fig. 1. Reversible bleaching of chlorophyll *in vivo*.

(decline of absorption at 710 to 715 mμ and increase at 730 mμ), as well as the bleaching at 475 mμ noted by Duysens, remain to be interpreted. Several reversible changes of chlorophyll may occur at once in the cell, for example, the formation of metastable triplet molecules may be superimposed on that of the semiquinone. It will be noted, however, that the effect observed at 475 mμ is opposite in sign to that expected from the formation of metastable chlorophyll-*a* (*20*).

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References and Notes

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18. We wish to thank R. Emerson and Mrs. R. Chalmers for supplying *Chlorella* cells.
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## Clot-Retraction Promoting Factor (Retractin) in Platelets and Tissues

Transfusion of platelet-rich blood or plasma in thrombocytopenic patients is followed by temporary elevation of the number of circulating thrombocytes and by temporary correction of the increased capillary fragility, the prolonged bleeding time, and the poor utilization of prothrombin during clotting that are typical of these patients (*1*). Isolated or preserved platelets fail to survive in the patient's circulation (they are, thus, nonviable), but their administration is equally followed by improvement in prothrombin consumption. This observation led to the isolation of the platelet thromboplastic factor (*2*).

It was observed in the course of unpublished studies that clot retraction also could be favorably influenced by administration of nonviable, preserved platelets. This finding prompted the present investigation (*3*) in which we discuss preliminary evidence for a platelet constituent that is responsible for clot retraction. This factor is referred to as "retractin."

The factor that promotes clot retraction was obtained from either lyophilized or fresh human or bovine platelets. Platelets were collected and separated by the multiple centrifugation technique previously described (*4*). Only preparations were used that contained no white or red cells by microscopic examination. Platelets were washed twice with saline solution at 4°C and packed by final centrifugation at 3500 rev/min for 30 min. Some aliquots were lyophilized by a standard technique, and other aliquots were used fresh.

Three different extraction techniques were used for the separation of retractin: (i) water-acetone extraction procedure; (ii) water-ethyl ether extraction procedure; and (iii) ethyl ether cold precipitation procedure.

1) To 6 mg of lyophilized platelets or to fresh platelets from 50 ml of fresh blood were added 2.5 ml of distilled water. After 24 hr at −20°C, the preparation was brought back to 4°C. Precooled acetone, 7.5 ml in volume, was then added. After it had been shaken for 5 minutes, the mixture was stored at −20°C for 12 hours; it was then centrifuged at 3500 rev/min for 30 min at 4°C to separate all particulate matter. Microscopic examination of the supernatant acetone for platelets, platelet fragments, and ghosts was negative. A 0.5 ml volume of supernatant acetone was then dried at room temperature under 29-in. vacuum aspiration. The dry material was suspended in 0.5 ml of saline solution.

2) To packed, fresh, washed platelets from 50 ml of fresh blood, 3 ml of precooled distilled water and 5 ml of ethyl ether were added. After centrifugation at 3500 rev/min for 30 min at 4°C, three well-differentiated layers were present: (i) an upper layer containing ethyl ether; (ii) a middle layer containing a precipitate, probably of protein material and stroma, and (iii) a bottom layer containing water. The ether layer was aspirated. Aliquots of 0.5 ml were dried at room temperature under 29-in. vacuum, and the dry material was resuspended in 0.5 ml of saline solution.

3) It was observed that storage at −20°C of ethyl ether containing platelets from 50 ml of fresh blood would be followed by formation of a precipitate, which would promptly redissolve at room temperature. The cold precipitate was washed with ethyl ether twice at −20°C; it was finally dissolved in 5 ml of ethyl ether. Aliquots of 0.5-ml were dried and then resuspended in 0.5 ml of saline at room temperature.

Retractin was assayed by the two following experiments. (i) Native platelet-poor human plasma was prepared as previously described (*4*). One milliliter of plasma was then added to 0.5 ml of saline suspension of the acetone or ethyl ether extracts in chemically clean glass test tubes. After mixing of the contents, the tubes were incubated in a water bath at 37°C, and the contents were allowed to clot; retraction was observed at various intervals of time. It occurred within 15 to 40 min in most samples that contained retracin (Fig. 1). (ii) A solution containing 300 mg percent of bovine commercial fibrinogen in saline solution and a solution containing 100 N.I.H. units of bovine thrombin per milliliter were first prepared. In glass test tubes incubated in a water bath at 37°C, 0.5 ml of saline suspension of acetone or ethyl ether extracts, 0.9 ml of fibrinogen solution, and 0.1 ml of thrombin solution were introduced in rapid succession. Clot retraction occurred after approximately 20 min in the samples that contained retractin. In addition to visual inspection, the activity of retractin preparation was evaluated with a semiquantitative technique. This consisted in the measurement of the volume of serum or saline solution expressed by the spontaneous retraction of the clot after 1 hr of incubation at 37°C. The ratio

$$\frac{\text{Volume of serum or saline expressed}}{\text{Total volume of mixture}} \times 100$$

was taken as an index of clot retraction (Table 1).

Clot retraction in test tubes containing native platelet-free plasma was absent or minimal; it never exceeded 10 to 12 percent in test tubes containing fibrinogen solution.

The two ethyl ether preparations were found to be free of thromboplastic factor. On the other hand, the acetone

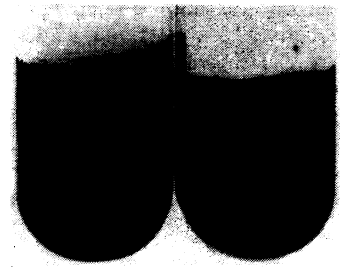

Fig. 1. Clot-retraction promoting effect of an acetone extract of human platelets (retractin) when added to native platelet-poor human plasma: (left) control tube containing 0.5 ml of saline solution; (right) tube containing 0.5 ml of acetone extract of platelets suspended in saline. Photograph was taken 2 hr after incubation of the test tubes in a water bath at 37°C.