with adenine as substrate was 1.0×10^{-5} for the wild type, and 0.8×10^{-5} for the mutant; and with PRPP as the substrate, the K_m was 3.4×10^{-4} for the wild type and 2.6×10^{-4} for the mutant. Both enzymes were equally sensitive to inhibition by inorganic pyrophosphate, 60 to 65 percent at $5 \times 10^{-3}M$ and 100 percent at $5 \times 10^{-2}M$, but with $5 \times 10^{-2}M$ sodium fluoride the mutant was only 44 percent inhibited as compared to 100 percent for the wild type. Heat-inactivation showed a greater temperature sensitivity for the mutant enzyme. At 55°C the extent of inactivation was twice that of the wild type. The time required for 50-percent inhibition was 48 seconds for the mutant enzyme compared to 96 seconds for the wild type. This twofold difference in the rates of inactivation of the two enzymes was consistent within the range studies 50° to 60°C and was not significantly altered by either a twofold increase or decrease of enzyme protein concentration. The *p*H for optimum activity was less for the mutant enzyme, 6.8 as compared with 7.6 for the wild-type enzyme. The effect of *p*H was more striking at *p*H 8.0 where wild-type activity remained near optimum but mutant activity was decreased to 47 percent of its optimum. As shown in Table 1, there is no difference in substrate activities, of the two enzymes with adenine as substrate compared to the complete loss of activity in the mutant when diaminopurine is the substrate. Another analog, 8-azaadenine, can be pyrophosphorylated by adenylic pyrophosphorylase and though the mutant retains this activity it is only 27 percent as active as the wild type. No activity for either preparation could be shown with 2-aminopurine, isoguanine (2-oxyadenine), hypoxanthine, guanine, xanthine, 6-mercaptopurine, or 8-azaguanine.

Thus, a new protein species has been created by the genetic alteration which gave rise to the mutant. It is also of interest to note, in terms of active sites, that what represents a mutant form in Salmonella may be the normal form in other organisms, for the adenylic pyrophosphorylase of yeast has been reported to be inactive in pyrophosphorylation with diaminopurine (*1, 6*).

GURUDUTT P. KALLE*
JOSEPH S. GOTS
Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia 4

8 NOVEMBER 1963

References and Notes

1. A. Kornberg, I. Lieberman, E. S. Simms, *J. Biol. Chem.* **215**, 417 (1955).
2. J. G. Flaks, M. J. Erwin, J. M. Buchanan, *ibid.* **228**, 201 (1957); L. N. Lukens and K. A. Herrington, *Biochim. Biophys. Acta* **24**, 432 (1957); C. E. Carter, *Biochem. Pharmacol.* **2**, 105 (1959); G. P. Kalle and J. S. Gots, *Federation Proc.* **20**, 358 (1961).
3. R. W. Brockman, C. S. Debavadi, P. Stutts, D. J. Hutchinson, *J. Biol. Chem.* **236**, 1471 (1961).
4. G. P. Kalle and J. S. Gots, *Biochim. Biophys. Acta* **53**, 156 (1961).
5. ———, *ibid.* **51**, 130 (1961); *Proc. Soc. Exptl. Biol. Med.* **109**, 277 (1962).
6. Supported by grants from the National Institutes of Health (CA-02790) and the National Science Foundation (G-5496).
* Present address: Biology Division, Atomic Energy Establishment, Trombay, Richardson, and Cruddas Building, Byculla, Bombay 8, India.

26 August 1963

Fluorescence and Absorption Changes in Chlorella Exposed to Strong Light: The Red Band

Abstract. *The reversible decrease in the apparent intensity of the main red absorption band of chlorophyll* a *at 680 mμ upon strong illumination of* Chlorella *cells, described by Coleman and Rabinowitch in 1957, is shown, by a polarization method, to be due to an increase in the fluorescence yield. True absorption changes are observed at 657 mμ (positive) and 648 mμ (negative).*

The steady-state difference spectrum of *Chlorella* (that is, the absorption of illuminated cells less the absorption of non-illuminated cells), measured in the red region by Coleman and Rabinowitch (*1, 2*) appears complex, with negative bands at 630, 650, 680, and 710 mμ appearing upon irradiation of a cell suspension with white light of high intensity. The difference spectrum of *Chlorella* measured by Kok (*3*) before and after flashes, also shows a complex structure in the red region, with negative bands at 650, 680, and 700 mμ. The prominent band at 680 mμ, noted in both papers, suggested reversible bleaching of chlorophyll *a* during photosynthesis (*2*).

When using a flow system, Strehler and Lynch (*4*) observed no absorption changes at 680 mμ upon irradiating *Chlorella* cell suspensions with light of low intensity. Strehler (*5*) suspected that the apparent absorption changes at 680 mμ, noted by others, were in fact changes in the yield of fluorescence due to the detecting beam, caused by actinic light. Fluorescence due to the actinic beam itself does not affect the measurements, because this light is constant; only the modulated light of the measuring beam, and the modulated fluorescence produced by it, cause a response in the detecting system.

The fluorescence yield of chlorophyll in *Chlorella* is known to increase by a factor of about 2 with increasing intensity of exciting light, when photosynthesis reaches saturation (*6*). The increase in fluorescence yield can thus appear as a negative change in absorption in the difference spectrum. This effect is proportional to the difference between the slopes of the curve "fluorescence intensity as a function of excitation intensity," in low light (measuring beam alone) and high light (measuring beam plus actinic beam). This slope has been shown by Brugger (*7*) to be constant at low intensities of exciting light, but increasing in steepness in the region where photosynthesis becomes light-saturated and approaching another constant value at high light intensity. This can explain the sigmoid shape of the light intensity dependence of the spectral change at 680 mμ, which was observed by Coleman and Rabinowitch (*1, 2*). Coleman (*8*) checked this hypothesis by rerouting his detecting beam so that it traversed the suspension at a right angle to its usual straight path, but concluded that changes in fluorescence due to the detecting beam contributed less than 7 percent to the changes measured at 680 mμ.

The more recent results presented here, however, suggest that an increase in the yield of fluorescence might have produced most, if not all, of the "negative absorption band" observed by Coleman at 680 mμ.

To measure the difference spectrum, we used an instrument developed in our laboratory from Duysens' original instrument (*9*), and similar in principle to that used in the study by Coleman and Rabinowitch. The monochromatic measuring beam (band width, 3.3 mμ), modulated by a rotating disk, was obtained from a 6-volt ribbon filament lamp and passed through a Bausch and Lomb 250-mm focal length grating monochromator, with the entrance and exit slits set at 0.50 mm. The cell suspension of *Chlorella pyrenoidosa* was irradiated at right angles to the detecting beam by blue light from another 6-volt ribbon filament lamp (filtered through Corning color filter No. 4-72). Red filters were placed in front of the detector (photomultiplier RCA 6217) to prevent the actinic light

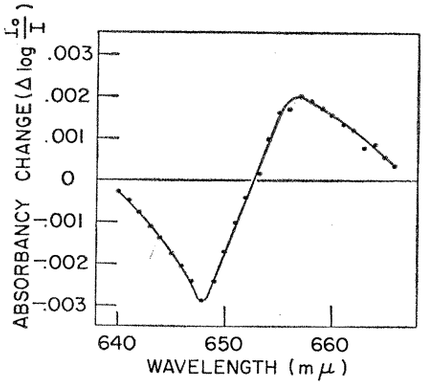

Fig. 1. The change in the absorption spectrum of a *Chlorella* suspension upon irradiation with blue light of high intensity (corrected for fluorescence).

from overloading the photomultiplier.

The changes in fluorescence due to the measuring beam were separated from absorption changes by two methods:

1) A narrow band interference filter, placed in front of the photomultiplier, was used to remove a large fraction of the fluorescence due to the measuring beam. A large fraction of the measuring beam passed this filter, which thus reduced the fluorescence effect compared with the absorption effect. Such an interference filter can be used effectively below 670 mμ; at 680 mμ its effectiveness is sharply reduced, since the fluorescence band itself has a peak at 680 to 690 mμ.

Results obtained by this method clearly show that a fluorescence effect is superimposed upon a positive absorption change at 657, and a negative absorption change at 648 mμ (Fig. 1). When Coleman's difference spectrum obtained in high-intensity light is corrected for the fluorescence effect, it agrees with these conclusions; and so do the observations of Strehler and Lynch (*4*) at low intensities of actinic light.

2) In the second method, we utilized the fact that the fluorescence in *Chlorella* is largely (97 percent) depolarized (*10*), while light transmitted in the forward direction is only slightly depolarized (5 percent). We introduced a polarizer in the measuring beam in front of the suspension, and an analyzer in the same beam between it and the photomultiplier. Measurements were made at a series of wavelengths, including 680 mμ, with the polarizer parallel to the analyzer and with the polarizer at 90° to the analyzer. Absorption changes should disappear (within the noise level of the instrument) on crossing the polarizer and the analyzer, while fluorescence changes should be reduced only by 3 percent. At 680 mμ the observed effect was not reduced markedly upon crossing the polarizers, suggesting that it is due predominantly or entirely to changes in fluorescence intensity. Measurements at shorter wavelengths gave results in agreement with those obtained with interference filters.

These results suggest that the 680-mμ band in the difference spectrum that appears in *Chlorella pyrenoidosa* in strong light, is not due to the absorption changes, but to changes in the yield of fluorescence caused by the measuring beam. This can happen not only in steady state measurements (with readings taken during the illumination), but also in "flash" or "flow" experiments where measurements are made in the dark after the flash, because, as Butler (*11*) has recently shown, the higher yield of fluorescence does not decrease immediately upon reduction of the intensity of exciting light, but decays slowly (for several seconds) until it reaches its steady value.

Experiments by Karapetyan, Litvin, and Krasnovsky (*12*), published while this paper was in preparation, suggest that not only the 680 mμ band in *Chlorella,* but also certain changes in the infrared region observed in the difference spectra of photosynthetic bacteria, are due to fluorescence.

DANIEL RUBINSTEIN
EUGENE RABINOWITCH
Department of Botany,
University of Illinois, Urbana

References

1. J. W. Coleman, A. S. Holt, E. Rabinowitch, in *Research in Photosynthesis,* H. Gaffron, Ed. (Interscience, New York, 1957), pp. 68–74.
2. J. W. Coleman and E. Rabinowitch, *J. Phys. Chem.* **63**, 30 (1959).
3. B. Kok, *Acta Botan. Neerl.* **6**, 316 (1957).
4. B. Strehler and B. Lynch, *Arch. Biochem. Biophys.* **70**, 527 (1957).
5. B. Strehler, in *Research in Photosynthesis,* H. Gaffron, Ed. (Interscience, New York, 1957), discussion on pp. 71–72.
6. Shiau and J. Franck, *Arch. Biochem. Biophys.* **14**, 253 (1947); P. Latimer, T. T. Bannister, E. I. Rabinowitch, in *Research in Photosynthesis,* H. Gaffron, Ed. (Interscience, New York, 1957), pp. 107–112.
7. J. E. Brugger, in *Research in Photosynthesis,* H. Gaffron, Ed. (Interscience, New York, 1957), pp. 59–67.
8. J. W. Coleman, *ibid.,* discussion on p. 73.
9. L. M. N. Duysens, *ibid.,* pp. 59–67.
10. W. Arnold, and E. S. Meek, *Arch. Biochem. Biophys.* **60**, 82 (1956).
11. W. Butler, *Biochim. Biophys. Acta* **64**, 309 (1962).
12. N. V. Karapetyan, F. F. Litvin, A. A. Krasnovsky, *Biofizika* **8**, 191 (1963).

1 July 1963

Technique for Sustaining Behavior with Conditioned Reinforcement

Abstract. *Pigeons were intermittently reinforced with food for pecking at one key. Concurrent pecking at a second key intermittently produced conditioned reinforcers (the set of stimuli that accompanied food reinforcement, but not the food). Under these conditions, responding on the second key was maintained indefinitely. Rates and patterns of responding on the second key were a function of the schedule of conditioned reinforcement.*

In the present experiment, pigeons could peck at either of two keys which were available concurrently. Pecks on one key intermittently produced food on a variable-interval schedule of reinforcement. Pecks on the second key intermittently produced all of the stimuli associated with food reinforcement on the first key but it was impossible for the birds to eat, because the food magazine was raised for one-half second, so there was not time enough for the birds to reach the food.

More specifically, pigeons, maintained at 80 percent of their free-feeding weight, performed daily in a standard, two-key experimental space (*1*). Each daily session terminated after 50 food reinforcements were obtained. A yellow light and a blue light were projected upon the left key and right key, respectively. Responses on the left key were reinforced on a 3-minute variable-interval schedule with a 4-second access to the food magazine. This was accompanied by (i) the sound of the solenoid-operated magazine, (ii) the illumination of a magazine light, (iii) the absence of the key lights, and (iv) the absence of the two house lights which normally illuminated the experimental space. Responses on the right key were reinforced on an independent 3-minute variable-interval schedule with all of the above stimulus changes occurring for a duration of one-half second.

Figure 1 shows a final performance, a stable state which could be maintained indefinitely, under this procedure, for two birds. The left records are cumulative response records of performance maintained by the variable-interval schedule of food reinforcement. A constant rate of responding appropriate to this schedule of reinforcement was sustained at approximately 1.5 to 2.0 pecks per second.