the regulatory mechanisms of the pathway. Several features of the repression phenomenon are discussed.

The authors wish to express their sincere appreciation to Miss Mary Lynn Gordon and Mr. Richard Timothy Eakin for their technical assistance.

* This investigation was supported by research grants GM 10166-01 and RG-6492 (C3) and training grant 2G-337 (C2) from the National Institutes of Health, U.S. Public Health Service. Published as Journal Paper No. 1595 of the North Carolina Agricultural Experiment Station.

[1] Armstrong, F. B., M. L. Gordon, and R. P. Wagner, these PROCEEDINGS, **49**, 322 (1963).

[2] Wagner, R. P., and A. Bergquist, *Genetics*, **45**, 1375 (1960).

[3] Armstrong, F. B., and R. P. Wagner, *Genetics*, **47**, 1581 (1962).

[4] Doudney, C. O., and R. P. Wagner, these PROCEEDINGS, **38**, 196 (1952).

[5] Wagner, R. P., and A. Bergquist, *J. Biol. Chem.*, **216**, 251 (1955).

[6] Gorini, L., and W. K. Maas, in *Chemical Basis of Development*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1958), p. 463.

[7] Freundlich, M., R. O. Burns, and H. E. Umbarger, these PROCEEDINGS, **48**, 1804 (1962).

[8] Umbarger, H. E., in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 301.

---

*ON THE LIGHT-INDUCED BLEACHING OF PHOTOSYNTHETIC PYRIDINE NUCLEOTIDE REDUCTASE IN THE PRESENCE OF CHLOROPLASTS**

BY BRITTON CHANCE AND ANTHONY SAN PIETRO†

JOHNSON RESEARCH FOUNDATION, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA, AND MCCOLLUM PRATT INSTITUTE, THE JOHNS HOPKINS UNIVERSITY, BALTIMORE

Communicated January 29, 1963

The recognition of the important function of the methemoglobin reducing factor of Davenport, Hill, and Whatley[1, 2] came with the studies of photosynthetic pyridine nucleotide reductase (PPNR)‡ by San Pietro and Lang.[3] These studies provided an important step in the delineation of the light-induced redox reactions of chloroplasts.[4]

As the preparation became purified to a degree sufficient for optical tests (it has recently been crystallized[5]), it became of interest to learn whether or not the bleaching of this pigment caused by dithionite could be achieved by illuminated chloroplasts and, if so, whether or not the rate of the light-induced bleaching is sufficient to explain the rate of NADP reduction. In brief, it was important to know whether or not the spectroscopically identifiable change is part of the action of PPNR. The importance of this work is underlined by a statement of Tagawa and Arnon[6] in 1962 on the bleaching of PPNR (renamed "chloroplast ferredoxin" by them): "Under none of these other names was its capacity demonstrated for undergoing reversible oxidoreduction accompanied by characteristic spectral changes." Our preliminary results on reversible spectral changes of PPNR were reported in 1960,[7] and the reversible bleaching was recently confirmed.[8, 9]

Preparations.—Chloroplasts were prepared as described previously[10] and the assay was carried out as described there. The concentration of PPNR is now based upon

a molecular weight of 17,000[11] and the concentration of the stock solution was found to be 359 μM on this basis as compared with 600 μM estimated in our preliminary report.[7] The optical path was 1 cm. The extent of bleaching was evaluated from the extinction coefficient of 2.6 cm^{-1} mM^{-1} for the wavelengths 480 and 540 mμ. The temperature of the experiments was 26°. The experiments were carried out in air-saturated sucrose-KCl medium[10, 12] and no phosphate acceptor system was added.

The experiments were carried out in the 2.7 ml cuvette of the double beam spectrophotometer. The reaction mixture consisted of 13.8 μM PPNR and 2.5 μg chlorophyll/ml, with and without 0.1 mM NADP. Red illumination was afforded by a tungsten lamp and a Corning 2403 filter.[13, 14] The photomultiplier was protected from the red illumination by Wratten 44A and Corning 9978 filters.

Experimental Results.—Difference spectra: Difference spectra for the light-dark condition were obtained by repetitive illuminations of the same sample of PPNR and by measuring the absorbancy changes at different wavelength settings of the double beam spectrophotometer.[13, 14] At each pair of wavelengths employed, the sensitivity toward absorbancy changes was the same [approximately 3 μM/one large scale division on the pen and ink recorder (see Fig. 2)]. The red light intensity at which the light-dark difference spectra were obtained was approximately one half that used in the kinetic experiment described below. This was done so that satisfactory measurements could be made at shorter wavelengths down to 370 mμ. Thus, the optical density changes in Figure 1*A* are less than those of Figure 3 (see Fig. 2).

The light-dark difference spectrum for the light illumination of 13.8 μM PPNR is shown in Figure 1*A*, and it is seen that a concentration change of approximately 1.1 μM is recorded; 8 per cent of the 13.8 μM PPNR is bleached under these conditions (cf. Fig. 3). Eleven per cent bleaching was reported in our preliminary report.[7] A difference spectrum obtained in this way shows the double peaks characteristic of that obtained with dithionite bleaching (see Fig. 1*B*).

Effect of light intensities: Figure 2 shows on a slow time scale typical responses of PPNR to illumination at various intensities of light. Although the recordings are not on a sufficiently fast time scale for high accuracy in rate recording, it is apparent that the rate of PPNR bleaching varies with the light intensity in the range of these experiments; the reaction rate is light-limited. This figure also served to indicate the light intensity used in these experiments; the spectrum of Figure 1*A* was obtained with intensity setting 5. The kinetics of Figure 3 were obtained with light intensity setting 6. (This intensity setting represented maximal voltage on the lamp.) The ratio of intensity setting 6 to 5 is 2.4-fold.

Kinetics of reduction: In Figure 3 we have recorded on a faster time scale and an expanded absorbancy scale the kinetics of reduction of the PPNR, and it is seen that the reaction starts almost immediately. The apparent initial rate is 0.27 μM/sec and the amplitude of the effect corresponds to 3.9 μM oxidized or 28 per cent of the 13.8 μM PPNR. In this case the activity of chloroplasts in reducing PPNR is measured directly, without added NADP. The rate corresponds to 390 μmoles PPNR/mg chlorophyll/hr at 26° and may be compared with the overall photosynthetic rate of 220 μmoles CO_2 fixed/mg chlorophyll/hour or 440 μmoles of a 2 equivalent substance reduced/mg chlorophyll/hr at 20°.

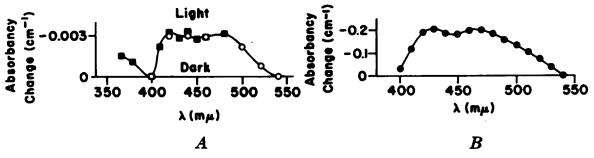

FIG. 1A.—Light and dark difference spectra for PPNR [13.8 μM illuminated in the presence of chloroplasts (2.5 μg chlorophyll/ml)].

FIG. 1B.—Difference spectrum for dithionite-reduced PPNR. 69 μM PPNR reduced with dithionite. The reference point for this spectra is 540 mμ.

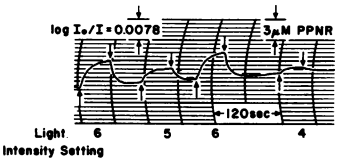

FIG. 2.—The effect of light intensity upon the extent of oxidation of PPNR. Steps 4, 5, and 6 correspond respectively to the intensity ratio 1:1.6:3.9 (Expt. 157).

Effects of NADP: In Figure 3B the kinetics of Figure 3A are repeated in the presence of 100 μM NADP. First, the steady state oxidation level is greatly reduced; second, the rate is somewhat reduced. In this experiment the apparent initial rate has fallen to 0.19 μM/sec, and in a companion experiment the rate fell from 0.33 to 0.15 μM/sec.

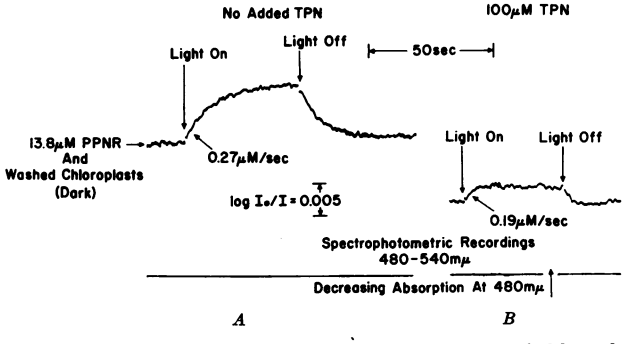

FIG. 3A.—Kinetics of bleaching of 13.8 μM PPNR in the presence of chloroplast (2.5 μg chlorophyll/ml) but in the absence of NADP. The time and absorbancy scales are indicated on the diagram. A decrease of absorbancy at 480 mμ with respect to 540 mμ corresponds to an upward deflection of the trace. The moments at which the lights turn on and off are marked on the figure.

FIG. 3B.—An experiment identical to that of Fig. 3A except that 100 μM NADP is present (Expt. 157).

Kinetics of reoxidation: It is a matter of considerable interest to note the rapidity of the dark oxidation of the reduced form of PPNR; the rates exceed the apparent initial rate of reduction, being 0.29 μM/sec in the absence of NADP and 0.23 in the presence of NADP. Thus, the true initial rate of reduction might exceed considerably the values indicated by the slopes of the traces of Figure 3. The oxidant is presumably molecular oxygen,[6] but our results are also consistent with a cyclic reaction.

Over-all activity in NADP reduction: The chloroplast-PPNR system was tested in the same concentration of NADP used in Figure 3, and the rate of NADP reduction measured at 340 mμ was 0.1 μM/sec. This value is one half the rate of PPNR reduction observed in the presence of NADP and one third the rate observed in the absence of NADP. The over-all activity corresponds to 140 μmoles NADP reduced/mg chlorophyll/hr at 26°.

Discussion.—Spectroscopic changes: The similarity of the light-induced absorbancy changes and those caused by the strong reducing agent, dithionite (Fig. 1), indicate a reduction of PPNR on chloroplast illumination. From a comparison of these spectra we calculate the percentage reduction of PPNR in the light-induced steady state to be 28 per cent. This percentage would be larger in the absence of the reaction which causes reoxidation of the reduced form of PPNR (see below).

The effect of NADP on the absorbancy change (Fig. 3) is larger than would be expected from the rate of NADP reduction, and it is possible that NADP combined with PPNR gives a reaction intermediate of a different absorption spectrum.

The spectral changes do not identify the moiety of PPNR that is reduced, although the iron atoms[6] are possible electron carriers. Moreover, a comparison of the spectra of the bacterial and chloroplast materials gives the ratios of the extinction coefficients of the 390 and 465 mμ bands as 5:1, and that of the iron contents as 5:1.[9] Furthermore, the shift of the peak of the band to shorter wavelengths with an increasing number of iron atoms is in agreement with observations of model iron compounds (J. B. Nielands, personal communication).

Reaction kinetics: The observation that PPNR is reduced more rapidly than NADP shows that the reduced form has the potentiality of acting as an electron carrier between chloroplasts and NADP. Similar data on the reaction kinetics of PPNR with hydrogen gas as the electron donor[6, 15] are needed to indicate the possibility that the reduced form (see Fig. 1) participates in the dark reduction of NADP.

Oxidation reaction: The very rapid reoxidation of reduced PPNR illustrated by Figure 3 casts some doubt on its capability for efficient biological function in reactions in which oxygen is present or is produced by light, as in chloroplasts. The kinetic data of Figure 3 suggest that PPNR rapidly donates electrons to an acceptor in the absence or in the presence of NADP. Thus, the efficiency of the NADP reduction must necessarily be low; if PPNR affords an electron "leak" to an alternate acceptor at this relatively rapid rate, high quantum efficiencies characteristic of photosynthesis are not to be expected under these conditions and more experimental data are needed.

The pathway of electron transfer: Based upon inhibition of the transhydrogenase by its antibody,[16] and upon studies of TPNH-cytochrome c reductase activity,[17, 18] the electron transfer and photophosphorylation pathway[19] appears to be

$$\rightharpoonup \text{cytochrome} \rightharpoonup \text{chlorophyll} \overset{\text{(PPNR)}}{\rightharpoonup} \text{Fe} \overset{\text{(TH)}}{\longleftrightarrow} \text{Fp} \leftharpoondown \text{TPN}$$
$$\swarrow \quad \searrow \quad \swarrow$$
$$\text{cytochrome c} \qquad O_2 \qquad (1)$$

Here, TH represents the transhydrogenase factor coupling PPNR to TPN,[17] which has also been purified by Shin *et al.*[20] The diagram also indicates the pathway of cytochrome c reduction[17] and possible pathways for the oxygen bypass discussed above.

Relation to ferredoxin: As stated in the introduction, the kinetics of the light-induced bleaching and the spectrum of the product are a matter of considerable interest to workers in this field.[1–9] Since the differences between the PPNR preparation used in these experiments and the material later identified by the name

"chloroplast ferredoxin" appear to be negligible, it is appropriate to identify the spectral and kinetic data obtained here with the function of methemoglobin reducing factor, PPNR, and chloroplast ferredoxin.

Relation to the mechanism of electron transfer in photosynthesis: The basic event in light-induced electron transfer in Chromatium is the donation of an electron from a cytochrome of type c to a form of chlorophyll, a reaction that has a high quantum efficiency[21] and is temperature-independent.[22] The nature of the primary electron acceptor is a matter of speculation, and PPNR or ferredoxin[23-27] is by no means the only possibility to be considered. In fact, the complication of its reaction with oxygen raises a further question of the possible efficiency of its function.

It is useful to point out that NAD reduction does not of itself require electron carriers of an oxidation-reduction potential as low as 432 mV[6]; NAD is reduced by interaction with a flavoprotein and ATP in the reversed electron transfer reaction of mitochondria.[28-29]

Summary.—Light-dark difference spectra for PPNR reduction by illuminated chloroplasts, together with the kinetics of reduction of the pigment and its reduction of NADP, show that the pigment is a permissible intermediate in the photosynthetic pyridine nucleotide reductase activity.

* This work was supported in part by the National Science Foundation.

† Present address: Charles F. Kettering Laboratories, Yellow Springs, Ohio.

‡ Abbreviations: PPNR, photosynthetic pyridine nucleotide reductase; NADP, triphosphopyridine nucleotide; μM, micromoles per liter; TH, transhydrogenase.

[1] Davenport, H. E., R. Hill, and F. R. Whatley, *Proc. Roy. Soc. (London)*, **B139**, 346 (1952).

[2] Davenport, H. E., and R. Hill, *Biochem. J.*, **74**, 493 (1960).

[3] San Pietro, A., and H. M. Lang, *J. Biol. Chem.*, **231**, 211 (1958).

[4] Davenport, H. E., in *Biological Structure and Function*, ed. T. W. Goodwin and O. Lindberg (New York: Academic Press, 1961), vol. 2, p. 449.

[5] Hill, R., and F. Bendall, *Nature*, **187**, 417 (1960).

[6] Tagawa, K., and D. I. Arnon, *Nature*, **195**, 537 (1962).

[7] Chance, B., in *Biological Structure and Function*, ed. T. W. Goodwin and O. Lindberg (New York: Academic Press, 1961), vol. 2, p. 453 (discussion of Arnon and Davenport).

[8] Horio, T., and T. Yameshita, *Biochem. Biophys. Res. Comm.*, **9**, 142 (1962).

[9] Whatley, F. R., K. Tagawa, and D. I. Arnon, these PROCEEDINGS, **49**, 266 (1963).

[10] Keister, D. L., A. San Pietro, and F. E. Stolzenbach, *Arch. Biochem. Biophys.*, **94**, 187 (1961).

[11] Appella, E., and A. San Pietro, *Biochem. Biophys. Res. Comm.*, **6**, 349 (1962).

[12] San Pietro, A., in *Light and Life*, ed. W. D. McElroy and B. Glass (Batimore: Johns Hopkins Press, 1961), p. 631.

[13] Chance, B. and B. Strehler, *Plant Physiol.*, **32**, 536 (1957).

[14] Chance, B., and R. Sager, *Plant Physiol.*, **32**, 548 (1957).

[15] Mortenson, L. E., R. C. Valentine, and J. E. Carnahan, *Biochem. Biophys. Res. Comm.*, **7**, **448** (1962).

[16] Keister, D. L., A. San Pietro, and F. E. Stolzenbach, *Arch. Biochem. Biophys.*, **98**, 235 (1962).

[17] Lazzarini, R. W., and A. San Pietro, *Biochim. et Biophys. Acta*, **62**, 417 (1962).

[18] Black, C. C., C. A. Fewson, M. Gibbs, D. L. Keister, and A. San Pietro, *Fed. Proc.*, **21**, 398 (1962).

[19] Keister, D. L., and A. San Pietro, in preparation.

[20] Shin, M., K. Tagawa, and D. I. Arnon, *Fed. Proc.*, **22**, 100 (1963).

[21] Olson, J. M., and B. Chance, *Arch. Biochem. Biophys.*, **88**, 40 (1960).

[22] Chance, B., and M. Nishimura, these PROCEEDINGS, **46**, 19 (1960).

[23] Witt, H. T., A. Muller, and B. Rumberg, *Nature*, **192**, 4806 (1961).

[24] Rumberg, B., A. Muller, and H. T. Witt, *Nature*, **194**, 854 (1962).
[25] Duysens, L. M. N., and J. Amesz, *Biochim. et Biophys. Acta*, **64**, 261 (1962).
[26] Crane, F. L., in *CIBA Foundation Symposium on Quinones in Electron Transport* (London, 1960), ed. C. E. W. Wolstenholme and C. M. O'Connor (London: J. & A. Churchill, Ltd., 1961), p. 36.
[27] Clayton, R. K., *Biochem. Biophys. Res. Comm.*, **9**, 49 (1962).
[28] Chance, B., and G. Hollunger, *Nature*, **185**, 666 (1960).
[29] Low, H., and I. Vallin, *Biochim. et Biophys. Acta*, **69**, 361 (1963).

PIG HEART GLUTAMIC ASPARTIC TRANSAMINASE MECHANISM OF TRANSAMINATION

BY A. E. EVANGELOPOULOS AND I. W. SIZER

DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

Communicated by F. O. Schmitt, March 20, 1963

Two principal mechanisms for the enzymatic transamination reaction have been proposed: (*A*) a discredited ternary mechanism in which it was assumed that interaction occurred simultaneously among all three reactants, amino acid, keto acid, and enzyme, at a single active site;[1-3] (*B*) the accepted binary hypothesis which proposed that enzymatic transamination is the sum of two separate binary reactions, each of which is catalyzed by a distinct form of glutamic aspartic transaminase (GAT), a pyridoxal form specific for an amino acid, and a pyridoxamine form specific for a keto acid in accordance with the following scheme:[4-6]

$$\text{amino acid}_1 + \text{pyridoxal} - \text{E} \rightleftharpoons \text{pyridoxamine} - \text{E} + \text{keto acid}_1$$
$$\text{keto acid}_2 + \text{pyridoxamine} - \text{E} \rightleftharpoons \text{pyridoxal} - \text{E} + \text{amino acid}_2$$
$$\text{Sum: amino acid}_1 + \text{keto acid}_2 \rightleftharpoons \text{amino acid}_2 + \text{keto acid}_1.$$

This scheme, however, does not satisfactorily explain the properties of the GAT and its reactions with keto substrates, amino substrates, and different types of inhibitors. The evidence against this binary hypothesis comes from the following observations:

1. The binary hypothesis does not explain why the spectrum of the dialyzed pyridoxal enzyme (prepared according to Jenkins *et al.*[7]) is different from the spectrum of the pyridoxal enzyme in the presence of the substrates, oxaloacetate, or α-ketoglutarate.[8] This distinct difference between enzyme and enzyme-keto substrate complex exists over a wide pH range. According to the binary scheme, the pyridoxal enzyme should not react with keto substrates. Evidence concerning complex formation between enzyme and substrate was obtained by studying the spectrum of pyridoxal enzyme (30 mg/ml or 0.00025 *M*) at pH 8.5 in 0.04 Tris buffer in the presence of oxaloacetate, α-ketoglutarate, aspartate, or glutamate (Fig. 1). Similar, but not identical, spectra are produced by adding the keto substrates, oxaloacetate, or α-ketoglutarate to the enzyme solution. The same is true for the comparable, but characteristically different, spectra produced by the amino substrates, aspartate, and glutamate. Spectroscopically, the final products and not intermediates are measured; hence, according to the binary scheme, the same spec-