INTRACELLULAR REACTIONS OF NICOTINAMIDE - DINUCLEOTIDE IN PHOTOSYNTHETIC ORGANISMS

J. AMESZ

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. W. DEN BOER, HOOGLERAAR IN DE FACULTEIT DER LETTEREN, TEN OVERSTAAN VAN EEN COMMISSIE UIT DE SENAAT TE VERDEDIGEN OP WOENSDAG 5 FEBRUARI 1964 TE 16 UUR

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PROMOTOR: PROF. DR. L. N. M. DUYSENS

STELLINGEN

I

In fotosynthetische algen en hogere planten vindt bij belichting een efficiënte reductie van nicotinamide-dinucleotide fosfaat plaats.

Dit proefschrift, hoofdstuk IV en V.

II

In tegenstelling tot de opvatting van Arnon en van Smith en French maakt chlorofyl *a* deel uit van beide fotosynthetische pigmentsystemen.

K.Tagawa, H.Y.Tsujimoto en D.I.Arnon, Nature, 199 (1963) 1247.
J.H.C.Smith en C.S.French, Ann. Rev. Plant Physiol., 14 (1963) 181.
L.N.M.Duysens, Proefschrift Rijksuniversiteit Utrecht, 1952.
Dit proefschrift, hoofdstuk IV.

III

De hypothese van Tollin dat het Emerson-effect kan worden verklaard door lichtabsorptie in het pigment P 700 wordt niet gesteund door de resultaten van experimenten.

G.Tollin, J. Theoret. Biol., 2 (1962) 105.

IV

Het gebruik van de term "flagellaat" ter aanduiding van de systematische positie van een organisme is onbevredigend.

R.Harder, in E.Strasburger, Lehrbuch der Botanik, 27. Aufl., Fischer, Stuttgart, 1958, p.342 e.v.

V

De methode van Keilin en Hartree voor de correctie van de extinctie van een strooiend medium berust op veronderstellingen die niet algemeen geldig zijn.

D.Keilin en E.F.Hartree, Biochim. Biophys. Acta, 27 (1958) 173.

VI

De conclusies van Harder en Bederke betreffende de groei van algen in licht van verschillende kleuren zijn onzeker door een onjuiste opzet en interpretatie van hun experimenten.

R.Harder en B.Berdeke, Archiv f. Mikrobiol., 28 (1957) 153.

VII

De door Vogel beschreven methode voor de jodometrische bepaling van tin biedt een te grote kans op onjuiste resultaten.

A.I.Vogel, A Text-book of Quantitative Analysis, 2nd ed., Longmans, London, 1955, p.352 e.v.

VIII

Voor de identificatie van fosfatiden kan niet worden volstaan met het toepassen van silicagel-chromatografie, gecombineerd met kleurreacties.

M.Kates, D.J.Kushner en A.T.James, Canad. J. Biochem. Physiol., 40(1962) 83.
U.T.M.Houtsmuller en L.L.M.van Deenen, Proc. Koninkl. Ned. Akad. Wetensch., B 66 (1963) 236.

IX

Aan de fotosynthese neemt ten hoogste slechts een klein gedeelte van het in de cel aanwezige plastochinon rechtstreeks deel.

J.Amesz, Biochim. Biophys. Acta, ter perse.

X

De door Molt en Tio aanbevolen methode van troebelheidsmeting van drinkwater is principieel onjuist.

E.L.Molt en L.H.Tio, Chem. Weekblad, 56 (1960) 553.

XI

Experimenten met celpreparaten geven slechts beperkte informatie over het mechanisme van de fotosynthese. Meer definitieve conclusies kunnen verkregen worden uit experimenten met intacte organismen.

XII

De resultaten van experimenten betreffende de invloed van het type auto op de bloeddruk van de bestuurder rechtvaardigen, in het belang van de verkeersveiligheid, uitgebreider onderzoek.

J. Koolhaas Revers, Autokampioen, 55 (1963) 3132.

XIII

De architectonische schoonheid van oude steden wordt meer bevorderd door conservatie, restauratie en reconstructie van gehele straten of wijken, dan van afzonderlijke gebouwen.

5 februari 1964

J. Amesz

kast dissertaties

Aan mijn ouders
Aan mijn vrouw

CONTENTS

CHAPTER I

INTRODUCTION

The overall process of photosynthesis may be considered as a light-induced reduction of CO_2 to carbohydrate and water and a concomitant oxidation of a hydrogen donor (ref. 215, p. 51):

$$CO_2 + 4\ HA \xrightarrow{h\nu} 1/n\ \left[CH_2O\right]_n + H_2O + 4\ A. \qquad 1.1$$

In photosynthesis of "green plants" (which means in this context all chlorophyll *a* containing organisms), the hydrogen donor is water, and the reaction reads:

$$CO_2 + 2\ H_2O \xrightarrow{h\nu} 1/n\ \left[CH_2O\right]_n + H_2O + O_2. \qquad 1.2$$

In photosynthetic bacteria other oxidizable compounds, e.g. H_2S or thiosulphate, act as hydrogen donors.

Since part of the light energy may also be used for the synthesis of compounds other than carbohydrates, the above equations only roughly approximate the nett result of the photosynthetic reactions. Moreover, as will be discussed in Chapter III, there are probably certain restrictions in applying equation (1.1) to photosynthetic bacteria.

During the past decades much effort has been made to study the mechanism of photosynthetic CO_2 reduction and to obtain information about the nature of possible intermediates in the reaction. It was found (127) that isolated illuminated chloroplasts from various species were able to evolve oxygen and to reduce mild oxidants, amongst other things potassium ferricyanide and certain dyes (see e.g. refs. 58, 134, 135, 259), but further progress was arrested because until more recently a sizable rate of reduction of CO_2 or of any plausible physiological intermediate was not obtained with cell-free systems of photosynthetic organisms.

In 1951, evidence for a light-induced reduction of NAD(P) by chloroplasts was reported by Vishniac and Ochoa (249) and, independently, by Tolmach (238) and Arnon (12), who obtained a (slow) reduction of NADP and NAD by an illuminated chloroplast preparation, under conditions where the reduced coenzyme was being taken away by an added substrate and enzyme system. As early as 1948 Krasnovskii (173, cf. ref. 44) reported a reduction of NAD in the light in the presence of ascorbic acid in a solution of chlorophyll in a mixture of pyridine and water, but these results were criticized recently by Bannister (26) and claimed to be an artifact. Studies, mainly by Calvin's group,

with C^{14}-labelled CO_2 indicated that the fixation and reduction of CO_2 in intact algae proceeds via the so-called reductive pentose phosphate cycle and that in this cycle probably 3-phosphoglyceric acid is the compound which becomes reduced in the light (27). The cycle could be kept going by means of $NADPH_2$ and ATP, which supported the hypothesis that a light-induced reduction of NADP would be an intermediate step in photosynthetic CO_2 reduction. The reduction of compounds other than 3-phosphoglyceric acid has also been postulated (153, 232), recently also by Calvin and coworkers (28, 30). Reviews on this subject are given by refs. 27, 29, 133, 232.

Sensitive absorption spectrophotometry of intact photosynthesizing cells gave at first conflicting evidence about the reduced intermediates of the photosynthetic reaction. In 1955 Duysens (69) observed an absorption increase in the ultra violet region upon illumination of suspensions of the green alga Chlorella and the red alga Porphyridium cruentum, which suggested a reduction of NAD or NADP in the light. However, Chance and coworkers obtained the opposite conclusion from experiments with a mutant of the green alga Chlamydomonas with abnormally low chlorophyll content (49, 50, see discussion in ref. 77), and did not obtain spectroscopic evidence for a reduction of NAD(P) in the purple bacterium Rhodospirillum rubrum (47). Studies of Duysens, Olson, Amesz and coworkers (74-76, 203-205) revealed that fluorescence spectroscopy provided a more specific and sensitive means for the identification of NAD or NADP and the study of reactions of these coenzymes *in vivo* and gave more conclusive evidence for a reduction of NAD(P) upon illumination of photosynthetic cells (Chlorella vulgaris, the blue-green alga Anacystis nidulans and the photosynthetic bacteria Chromatium strain D and Rhodospirillum rubrum).

San Pietro and Lang, in 1956, were the first to report a rapid accumulation in the light of NADP and NAD by isolated chloroplasts to which these coenzymes were added (220). It was found that the addition of a chloroplast extract (containing "PPNR", see Chapter V) was needed for the reduction. These results were soon confirmed by others and the *in vitro* reduction of NAD and NADP by chloroplasts of green plants and chromatophores of purple bacteria is being studied in several laboratories.

In the past photosynthesis proper was usually assumed to be driven by only one "primary" light reaction. Mechanisms of two or more different primary light reactions had occasionally been proposed and discussed earlier (see ref. 215, p. 150 and ref. 100), but direct information about the existence and mechanisms of two different light reactions in green plant photosynthesis was only obtained recently by means of sensitive spectrophotometry of photosynthesizing cells (168, 169, 80-82, 6). The results of these studies indicated that the two light reactions were driven by two different pigment systems with different pigment content and absorption characteristics and that the cooperation of both systems was needed to achieve green plant

photosynthesis. The two systems were called system 1 and 2 (80, 81). In Chapter IV evidence will be presented that system 1 effects an efficient reduction of NAD(P) in photosynthetic cells upon illumination. As will be discussed, bacterial photosynthesis is probably driven by only one pigment system, equivalent to system 1 in green plants.

In this thesis the results will be presented of a quantitative study on light-induced NAD(P) reduction. Most experiments were performed with intact cells of photosynthetic microorganisms by means of sensitive absorption and fluorescence spectrophotometry. The effect of illumination with light of different intensities and wavelengths and the effect of variation of other experimental parameters on the intracellular rate of coenzyme reduction or oxidation was examined and compared to the rate of cytochrome oxidation and oxygen evolution. Part of the results have also been published elsewhere (4, 6, 7).

CHAPTER II

MATERIALS AND METHODS

2.1 *Culturing of Algae and Bacteria*

The algae and bacteria used in our experiments were grown in liquid culture media. The cultures were obtained by inoculation from agar slants. Each time the suspension had reached its maximal density, which usually took a few days, it was diluted approximately 20-fold with fresh growth medium. Unless otherwise stated the culture medium was contained in cylindrical glass vessels, of 31 cm height and 35 mm inner diameter, and 200 ml capacity. They were of similar design as those used in Pirson's laboratory (162), except that no rubber or plastic tube joints were applied, so that the medium was in contact with glass only. From a side tube a gas stream, usually a mixture of air or N_2 and CO_2, was bubbled at a rate of 15 to 30 ml/min through the suspension. This prevented exhaustion of CO_2 and minimized pH changes and settling of the suspension, and, for purple bacteria, served to maintain anaerobiosis. The gas was filtered by means of a sterile cotton plug. The culturing vessels were covered by aluminium caps to prevent contamination.

The culturing tubes were thermostated in lucite (perspex) water baths. Illumination was effected from the side; algae were illuminated by means of fluorescent tubes, bacteria by means of incandescent lamps.

Unicellular organisms were harvested by centrifugation, usually at 1000 x g during 10 to 15 min and resuspended, usually in fresh medium, before measurement. The concentrations of the suspensions were measured by centrifugation in a Trommsdorff tube, and are given as volume percent of packed (wet) cells.

2.2 *Optical Effects in Suspensions*

Measurement of the absorbancy or absorptancy of biological material, such as suspensions of microorganisms, in practice presents various difficulties. Since the light usually is not only absorbed, but also scattered by the sample, a true absorption spectrum is only obtained when all scattered radiation is collected (see e.g. ref. 215, p. 672 and ff., refs. 101 and 164) or when proper corrections for the effect of scattering are applied (5, 178). Even then, for the quantitative evaluation of such a spectrum a further correction may be necessary, because inhomogeneous distribution of the pigments causes a distortion of the spectrum, compared to that of the same material homogeneously dispersed in solution (70, 5). In the following we will

mainly discuss effects which occur when small absorption changes and spectra of these changes are measured in suspensions of colored, scattering particles in transparent medium.

2.2.1 Scattering flattening

The measured absorbancy of a light-scattering suspension of particles depends strongly upon the way of measurement. When the absorbancy is measured by means of a light detector which is small and situated at a large distance from the absorption vessel, relatively much scattered radiation is lost, so that a too high absorbancy is measured and the spectrum is shifted upwards. It has been shown (5) that, for a dilute suspension, the spectrum which is measured when only the light transmitted within a small angle is caught is not only shifted, but also flattened with respect to a spectrum measured at a large angle.

The following relation, based upon certain assumptions, which in principle gives a correction for the effect of scattering, was obtained for not too concentrated suspensions (5):

$$E(180^\circ) \approx E'(\gamma) = \left[E(\gamma) - E''(\gamma)\right] / \left[1 - E''(\gamma)/p \log e\right]. \quad 2.1$$

In this equation $E(180^\circ)$ is the absorbancy which would be measured if all scattered radiation would be collected. As discussed in ref. 5, for a dilute and weakly scattering suspension $E(180^\circ)$ approximately equals the absorbancy which would be measured when no scattering occurred at all. $E'(\gamma)$ is the corrected absorbancy, $E(\gamma)$ is the absorbancy of the suspension as measured by means of a device which catches only light with a deviation from the incident beam of less than the angle γ. $E''(\gamma)$ is the absorbancy which would be measured if no intrinsic absorption took place within the particles, but the same scattering occurred as with the real suspension; e is the base of the natural logarithms and p is a constant equal to the total area of all particles in the light beam projected on a plane perpendicular to this beam, divided by the illuminated area of the vessel; p is thus proportional to the concentration of the suspension.

The scattering correction given by equation (2.1) can also be applied to the small absorption changes occurring in photosynthetic cells. When one assumes that no simultaneous scattering changes occur (for most suspensions there are no indications contrary to this assumption), $E''(\gamma)$ remains the same during an experiment, and the following equation is obtained:

$$\Delta E'(\gamma) = \Delta E(\gamma) / \left[1 - E''(\gamma)/p \log e\right]. \quad 2.2$$

In words: the corrected absorbancy difference, $\Delta E'(\gamma)$, is larger than that actually measured, $\Delta E(\gamma)$, since the latter value has to be divided by a number smaller than unity. This correction factor can be calculated in principle by means of equation (2.1). When the number, size and shape of the bacterial or algal cells are known, p can be calculated. $E(\gamma)$ is obtained by measuring

the absorbancy of the suspension against a blank in the absorption difference spectrophotometer. As will be discussed in §2.3, $E'(\gamma)$ can be determined with good precision in a sufficiently sensitive conventional spectrophotometer by measuring the absorbancy with a piece of opal glass placed behind the sample and reference cuvette. By means of equation (2.1), $E''(\gamma)$ and the correction factor $1 - E''(\gamma)/p \log e$ can now be calculated.

As will be discussed in §3.2 the correction can also be obtained in a more direct way, but only for relatively large absorbancy changes.

2.2.2 Particle flattening

Duysens (70) has shown that the absorbancy of a suspension of colored particles, even when corrected for scattering, is still lower than the absorbancy of a solution of the same pigments. This effect of "particle flattening" (also called "sieve effect") was found to depend upon the absorptancy of a single particle; the effect is strongest in spectral regions where strong absorption occurs, so that a distortion of the absorption spectrum results near the absorption maxima of the pigments. Application of the relations obtained to the absorption spectrum of Chlorella pyrenoidosa indicated a satisfactory agreement between the predicted and measured effects (70). More recently experimental support and somewhat extended calculations were given by studies of Latimer and Eubanks (178), of Tyuma *et al.* (234) and of Itoh *et al.* (145) on the absorption spectra of erythrocytes and spinach chloroplasts and chloroplast fragments.

For the quantitative evaluation of small absorbancy changes occurring in such suspensions, it may be even more important to take into account the effect of particle flattening, because, as we will see, the effects are relatively larger in absorbancy difference spectra than in "ordinary" absorbancy spectra.

For the (hypothetical) case of a not too dense suspension of cubical, oriented, homogeneously colored particles, the following relation was derived (70):

$$E'(\gamma) = (p \log e)\left[1 - T'_p(\gamma)\right]. \qquad 2.3$$

E_{sol}, the absorbancy of a solution of the same amount of pigments is given by:

$$E_{sol} = p \log \left[1/T'_p(\gamma)\right] \qquad 2.4$$

so that

$$E'(\gamma)/E_{sol} = (\log e)\left[1 - T'_p(\gamma)\right] / \log \left[1/T'_p(\gamma)\right]. \qquad 2.5$$

In words: the absorbancy of a suspension $E'(\gamma)$ is obtained by multiplying the absorbancy of the pigments in solution E_{sol} with a factor, which depends upon the transmittancy $T'_p(\gamma)$ of a particle. The factor approaches unity when $T'_p(\gamma)$ approaches unity, and

approaches zero slowly when $T'_p(\gamma)$ approaches zero. Thus the flattening is strong for strongly absorbing particles. It is independent of the concentration.

We define the flattening factor for small absorption changes (or "differential flattening") in a suspension as the quotient $\Delta E'(\gamma)/\Delta E_{sol}$ for a small variation of $T'_p(\gamma)$. Since both $\Delta E'(\gamma)$ and ΔE_{sol} are small, in first approximation for cubical particles:

$$\frac{\Delta E'(\gamma)}{\Delta E_{sol}} = \frac{dE'(\gamma)}{dE_{sol}} = \frac{d\{(p \log e)\ [1 - T'_p(\gamma)]\}}{d\{p \log [1/T'_p(\gamma)]\}} = T'_p(\gamma). \qquad 2.6$$

In words: the particle flattening factor of absorbancy changes in a suspension of cubical particles is equal to the transmittancy of a single particle, corrected for scattering.

For a suspension of homogeneously colored spheres, a similar calculation applies. According to Duysens (70, equation (18) and ff.):

$$E'(\gamma) = (p \log e)\left[1 - T'_{p\ av}(\gamma)\right] \qquad 2.7$$

where $T'_{p\ av}(\gamma)$, the average transmittancy of a sphere, is equal to:

$$T'_{p\ av}(\gamma) = 2\left[1 - (1 + \alpha_p)\, e^{-\alpha_p}\right]/\alpha_p^{\,2}. \qquad 2.8$$

$\alpha_p = \ln I_o/I = (\log e)\, E_p$; E_p is the absorbancy of a particle for a beam passing through the center of the sphere. Further:

$$E_{sol} = (2/3)\ p\ (\log e)\ \alpha_p. \qquad 2.9$$

These equations do not take into account refractions and reflections at the surface of the spheres; the refractive indices of the spheres and the surrounding medium are assumed to be equal. After substituting $T'_{p\ av}(\gamma)$ from equation (2.8) into (2.7), we obtain:

$$\frac{dE'(\gamma)}{dE_{sol}} = \frac{3}{2} \cdot \frac{d\left\{1 - 2\left[1 + (1 + \alpha_p)\, e^{-\alpha_p}\right]/\alpha_p^{\,2}\right\}}{d\alpha_p}, \text{ or}$$

$$dE'(\gamma)/dE_{sol} = 3\left[2 - e^{-\alpha_p}(2 + 2\alpha_p + \alpha_p^{\,2})\right]/\alpha_p^{\,3}. \qquad 2.10$$

The equations (2.10) and (2.8) enable us to calculate for each α_p the corresponding flattening $dE'(\gamma)/dE_{sol}$ and $T'_{p\ av}(\gamma)$. Fig. 2.1 gives the graphical relation between the differential flattening for oriented cubes (3) and spheres (4) and the transmittancy of a single particle. For comparison the corresponding curves (1) and (2) for the "ordinary" absorbancy of a suspension are also given. It appears that the flattening of an absorbancy difference spectrum is, for not too low transmittancy values, roughly twice as large as for an absorbancy spectrum; so a

correction for the first spectrum is even more important than for the latter one.

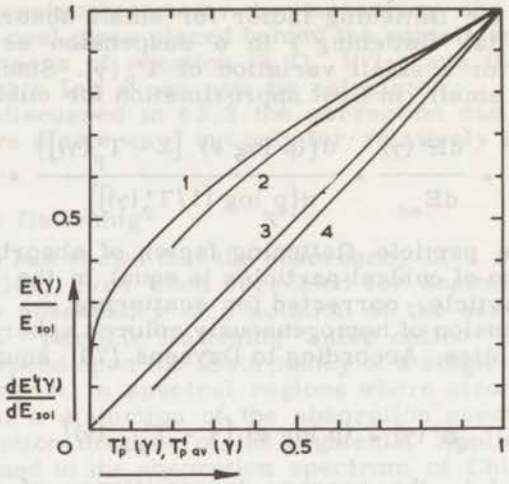

Fig. 2.1 "Particle flattening" of the absorbancy of a suspension of colored particles in transparent medium. Curve (1), plotted from equation (2.5), gives $E'(\gamma)/E_{sol}$, the ratio between the absorbancy of a suspension of oriented colored cubes and the absorbancy of a solution of the same amount of pigments, as a function of $T'_p(\gamma)$, the transmittancy of a single cube. Curve (2), obtained from Fig. 1 of ref. 70, gives $E'(\gamma)/E_{sol}$ for a suspension of spheres as a function of $T'_{p\ av}(\gamma)$, the average transmittancy of one sphere. Curves (3) and (4) apply for small absorbancy differences (e.g. as between an illuminated and non-illuminated suspension of photosynthetic organisms). The ratio between the absorbancy difference in a suspension and the corresponding one in solution, $dE'(\gamma)/dE_{sol}$ is given by curve (3) for a suspension of cubical particles as a function of $T'_p(\gamma)$ and by curve (4) for spheres as a function of $T'_{p\ av}(\gamma)$, plotted by means of equation (2.6) and (2.10) respectively. As discussed in the text, effects of light scattering are neglected.

According to equation (2.7), $T'_{p\ av}(\gamma)$ can for spherical particles be determined by measuring $E'(\gamma)$ and p, so that by means of Fig. 2.1, curve (4), the measured absorbancy changes can be corrected and converted to the corresponding absorbancy differences for a solution of the pigments. From the latter values the amount of reacting compound can be calculated, if its specific extinction coefficient is known.

2.2.3 Deviations from Beer's law

For a non-scattering suspension, Beer's law applies up to relatively high concentrations (70). However, if strong light scattering occurs, a serious deviation from Beer's law may occur if the suspension is not sufficiently dilute. In such a suspension a light ray is repeatedly scattered by the particles before

leaving the absorption vessel. When a large part of the scattered light is collected in the absorbancy measurement, and when the suspension consists of strongly absorbing particles, the measured absorbancy at high concentration is higher (cf. refs. 46, 157) than that expected from Beer's law, because the average light path is longer than the depth of the vessel. On the other hand, the opposite effect may be measured, when only a small part of the scattered radiation is caught, and when the transmittancy of the particles is high, because at high concentration part of the multiply scattered light is scattered back on the photocell.

Since equation (2.1) does not rigorously apply for a suspension in which repeated scattering occurs (5), the correction for "scattering flattening" becomes more uncertain for a too concentrated suspension.

2.3 *Absorbancy Measurement*

The absorbancy of suspensions of algae, bacteria or spinach chloroplasts was measured by means of a Zeiss PMQ II spectrophotometer. In order to minimize the effect of scattering, opal glass was placed behind both blank and sample vessel, according to the method devised by Shibata (223). An approximate correction for scattering was applied by subtracting from the measured absorbancy the apparent absorbancy at a wavelength where no intrinsic absorption occurs (740 mμ for algae and chloroplasts, 960 mμ for purple bacteria). Inspection of equation (2.1) shows that this correction implies two approximations: (1) $E''(\gamma)$ at any wavelength is equal to $E(\gamma)$ at 740 or 960 mμ and (2) $1 - E''(\gamma)/p \log e$ is equal to unity. The latter approximation gives an error which is probably not larger than 1% for a spectrum measured with opal glass (5); the error introduced by the first approximation may be larger since the amount of scattering and thus $E''(\gamma)$ may vary with wavelength.

Since the method of subtracting is a convenient and commonly used way to correct for scattering, a description of the following experiments, which render information about its validity and accuracy, may be useful. Latimer and Eubanks (178) devised a more refined way to correct for scattering. From equation (6) of ref. 5 (from which equation (2.1) was also derived), they obtained the relation:

$$E'(\gamma) = E(\gamma_1) - A\left[E(\gamma_2) - E(\gamma_1)\right], \qquad 2.11$$

where A is equal to:

$$A = \frac{1 - T''_p(\gamma_1)}{\left[1 - T''_p(\gamma_2)\right] - \left[1 - T''_p(\gamma_1)\right]}$$

$T''_p(\gamma)$, the apparent transmittancy of a particle if only scat-

tering occurred, is defined analogously as $E''(\gamma)$ (§ 2.2.1, and $T'_p(\gamma)$ (§ 2.2.2). Latimer and Eubanks assumed the amount of light scattered by a particle between the angle γ_1 to 180° to have the same wavelength dependence as that between γ_2 and γ_1. Then A is independent of wavelength, and from equation (6) of ref. 5 it can be calculated that:

$$A = \left[\frac{E(\gamma_1)}{E(\gamma_2) - E(\gamma_1)} \right] \lambda_o ,$$

λ_o being an arbitrary wavelength where no absorption and only scattering occurs. The various symbols are defined in § 2.2.1. By means of equation (2.11) Latimer and Eubanks calculated the corrected absorbancy spectrum ($E'(\gamma)$ spectrum) of suspensions of chloroplasts and red blood cells from the $E(\gamma_1)$ and $E(\gamma_2)$ spectra, measured with and without opal glass, respectively.

Since the assumption of Latimer and Eubanks is, at best, a good approximation, we have tested the method for suspensions of Chlorella ellipsoidea and Rhodospirillum rubrum (strain 1, grown in malate medium, see § 3.2) by measuring the absorbancy spectrum at three different angles ($\gamma_1 = 75°$, $\gamma_2 = 29°$, $\gamma_3 = 16°$). In each of two compartments of the vessel carrier of the Zeiss PMQ II spectrophotometer two diaphragms were placed. Diaphragms with a circular hole of 3 mm diameter, which served to narrow the measuring beam, were placed immediately in front of the 1 mm sample and reference cuvettes. Diaphragms with a circular hole of 10 mm were placed immediately in front of sheets of opal glass and at a certain distance behind the vessels. The distance between these diaphragms and the cuvettes and, consequently, the solid angle in which the scattered light was collected, was varied by moving the cuvettes and the first diaphragms, without changing their relative position.

Figs. 2.2 and 2.3 show the spectra obtained for three different angles and the correction $A[E(\gamma_n) - E(\gamma_1)]$ for two different values of γ_n. A was calculated from the absorbancies at 740 or 960 mμ. It can be seen that the corrections calculated from $E(\gamma_2)$ and $E(\gamma_1)$ and from $E(\gamma_3)$ and $E(\gamma_1)$ agree well, but it is also clear that the correction, for the range of wavelengths measured, does not much deviate from the value of $E(\gamma_1)$ at 740 or 960 mμ respectively, so that the error which is introduced by subtracting the latter value instead of the correction of Latimer and Eubanks is relatively small. For Chlorella there is a small, but presumably significant difference between the spectra obtained in these two ways; for Rhodospirillum no significant difference is obtained. The corrected spectrum of Chlorella was also calculated from $E(\gamma_3)$ and $E(\gamma_2)$. As the dashed line shows there is a fairly good agreement with the other spectra.

These observations indicate that the method of Latimer and Eubanks can be used to correct the absorption spectrum of unicellular photosynthetic organisms for errors caused by scat-

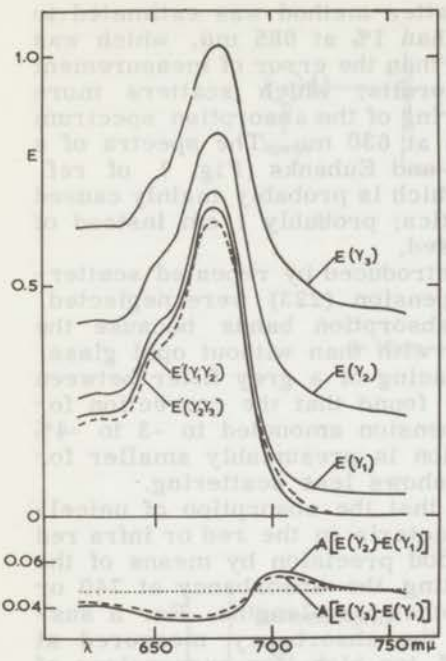

Fig. 2.2

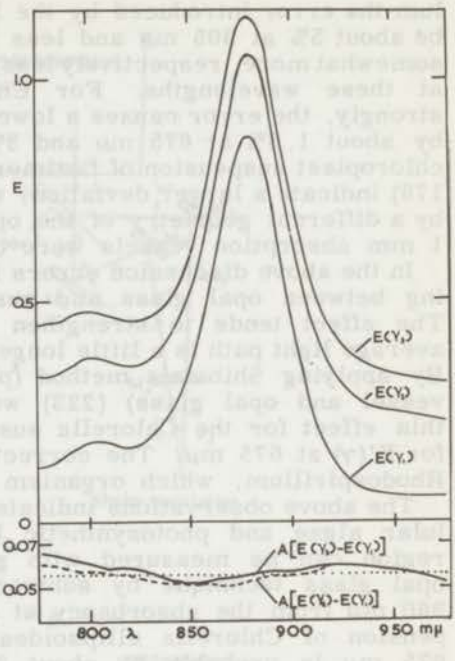

Fig. 2.3

Fig. 2.2 The absorbancy of a suspension of Chlorella ellipsoidea, measured with opal glass in a 1 mm absorption vessel as described in the text. The spectra $E(\gamma_1)$, $E(\gamma_2)$ and $E(\gamma_3)$ were measured with a distance of 0, respectively 8 and 16 mm between the absorption vessel and opal glass. The measurements were done at 2.5 mμ intervals between 640 and 720 mμ and at 5 mμ intervals at shorter and longer wavelengths; the half-width of the measuring beam was about 4 mμ between 620 and 720 mμ. The spectra $E'(\gamma_1\gamma_2)$ and $E'(\gamma_2\gamma_3)$ give the corrected spectra, calculated by means of equation (2.11) from $E(\gamma_1)$ and $E(\gamma_2)$, or $E(\gamma_2)$ and $E(\gamma_3)$, respectively. In the bottom part of the figure, the solid and dashed line give, on a different scale, the corrections for the $E(\gamma_1)$ spectrum, $A[E(\gamma_2) - E(\gamma_1)]$ and $A[E(\gamma_3) - E(\gamma_1)]$, respectively. The dotted line gives, for comparison, the value of $E(\gamma_1)$ at 740 mμ (see text).

Fig. 2.3 Absorbancy spectra of a suspension of Rhodospirillum rubrum, measured in the same way as in Fig. 2.2. The bottom part of the figure gives, on a different scale, $A[E(\gamma_2) - E(\gamma_1)]$ (dashed line), $A[E(\gamma_3) - E(\gamma_1)]$ (solid line) and the value of $E(\gamma_1)$ at 960 mμ (dotted line). The symbols have the same meaning as in Fig. 2.2.

tering. On the other hand our measurements indicate that the more convenient and simple way of correcting the opal glass spectrum by subtracting the absorbancy measured at a region where no pigment absorption occurs gives results which are as accurate or only little less so. For the spectrum of Rhodospiril-

lum the error introduced by the latter method was estimated to be about 5% at 805 mμ and less than 1% at 885 mμ, which was somewhat more, respectively less, than the error of measurement at these wavelengths. For Chlorella, which scatters more strongly, the error causes a lowering of the absorption spectrum by about 1.5% at 675 mμ and 3% at 630 mμ. The spectra of a chloroplast suspension of Latimer and Eubanks (Fig. 1 of ref. 178) indicate a larger deviation, which is probably mainly caused by a different geometry of the optics; probably 1 cm instead of 1 mm absorption vessels were used.

In the above discussion errors introduced by repeated scattering between opal glass and suspension (223) were neglected. The effect tends to strengthen absorption bands because the average light path is a little longer with than without opal glass. By applying Shibata's method (placing of a grey filter between vessel and opal glass) (223) we found that the correction for this effect for the Chlorella suspension amounted to -3 to -4% for $E'(\gamma)$ at 675 mμ. The correction is presumably smaller for Rhodospirillum, which organism shows less scattering.

The above observations indicate that the absorption of unicellular algae and photosynthetic bacteria in the red or infra red region can be measured with good precision by means of the opal glass technique by subtracting the absorbancy at 740 or 960 mμ from the absorbancy at other wavelengths. For a suspension of Chlorella ellipsoidea the absorbancy measured at 675 mμ is probably by about 2% too high; for suspensions of smaller organisms, as e.g. Rhodospirillum rubrum or Anacystis nidulans, which cause less light scattering (cf. ref. 5), the error near the absorption maxima is in general probably lower.

2.4 *Measurement of Absorbancy Changes*

2.4.1 Apparatus

In order to measure the small changes of absorbancy, occurring upon illumination of intact photosynthetic cells, a sensitive absorption difference spectrophotometer was constructed. The apparatus was of the "split-beam" type, earlier applied by Duysens (67, 72), and is illustrated in Fig. 2.4.

The light of a 6 V, 17 A tungsten ribbon filament lamp, operated on batteries, passed a Bausch and Lomb grating monochromator (f/4.4, 500 mm focal length, 1200 lines/mm grating). Half of a quartz disc (rd) driven by a synchronous motor, and rotating at a speed of 50 r.p. sec, was coated with a reflecting aluminium layer (Balzers, Liechtenstein, Alflex A). Thus the beam leaving the monochromator was reflected and transmitted alternatively during 0.01 sec periods. After passing the lens L_1 and the mirrors M_1 and M_3 and L_2 and M_2 respectively, both beams were reflected upwards by means of mirror M_4, so that both lay in the same vertical plane. Each beam passed an absorption

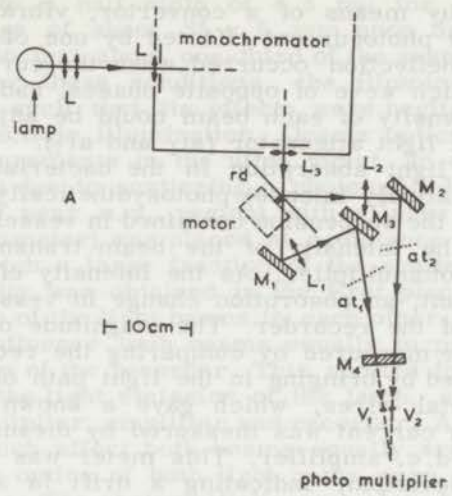

Fig. 2.4 A

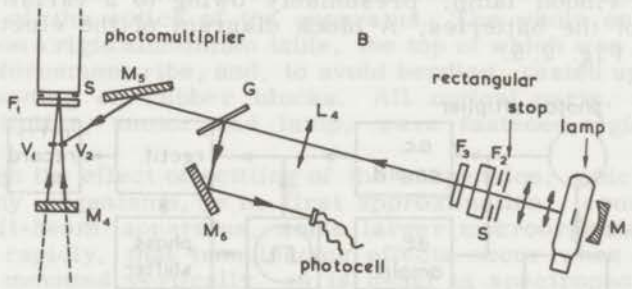

Fig. 2.4 B

Fig. 2.4 Schematic diagram of the apparatus for measuring light-induced absorption changes. L are lenses, M are mirrors, F are filters, S are shutters and V are absorption vessels; rd is the light chopper, a rotating sectioned disk, at are adjustable light attenuators, G is a glass plate. In Fig. A and B, the parts of the light path that lie in a horizontal and in a vertical plane, respectively, are given by solid lines.

vessel filled with suspension, a filter or combination of filters F_1, and finally hit the light-sensitive surface of the photomultiplier. By means of the lenses L_1 and L_2, both of the same focal length, an image of the exit slit of the monochromator was focussed upon the two absorption vessels. The mirrors M_1, M_2 and M_3 were placed in such a position that both light paths were of the same length.

The signal of the photomultiplier was fed into an a. c. amplifier, rectified, and recorded by means of a $\frac{1}{4}$ sec Honeywell-Brown strip-chart recorder, model 135X16V-X-157P9R. The rectification

was effected by means of a convertor, vibrating at 50 Hz in phase with the photocurrent effected by one of the two beams. No recorder deflection occurred when the currents due to the two beams (which were of opposite phases) had the same magnitude; the intensity of each beam could be adjusted separately by means of a light attenuator (at l_1 and at l_2).

Changes of light absorption in the bacterial or algal cells, caused by (relatively intense) photosynthetically active (actinic) illumination of the suspension contained in vessel V_2, will cause a change in the intensity of the beam transmitted by V_2 and hitting the photomultiplier. As the intensity of the other beam remains constant, an absorption change in vessel V_2 results in a deflection of the recorder. The magnitude of the absorption change could be measured by comparing the recorder deflection with one, caused by bringing in the light path of the right beam three thin metal wires, which gave a known weakening. The total multiplier current was measured by means of a μA meter connected to a d.c. amplifier. This meter was read at frequent intervals; a deviation, indicating a drift in sensitivity of the apparatus, was corrected by varying the multiplier voltage. A drift of sensitivity was often observed after switching on the tungsten ribbon lamp, presumably owing to a variation of the voltage of the batteries. A block diagram of the electronics is given in Fig. 2.5.

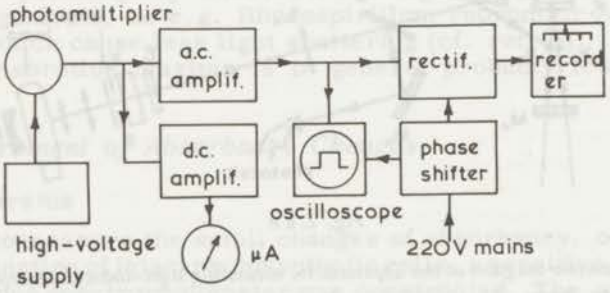

Fig. 2.5 Block diagram of the electronics of the absorption difference spectrophotometer.

As the intracellular reactions studied caused only small absorbancy changes both a high sensitivity and a high stability of the apparatus were required. A high sensitivity was obtained by the use of high-aperture optics, including a high-aperture monochromator. The Bausch and Lomb f/4.4 monochromator had a transmittance of 41% at 365, 48% at 436, 21% at 645 and 9% at 840 mμ. On the other hand, the absorption changes induced by the measuring beams should be as small as possible, and under normal conditions negligible compared to that caused by the actinic illumination. To obtain a not too high intensity at the cuvettes, an enlarged image of the exit slit was projected upon the vessels. Unless a better spectral resolution was desired, the apparatus

was routinely operated with slits of 20 mm high and 3 mm wide (corresponding to a half-width of 4.5 mμ for the measuring beam). An image of about 35 x 5 mm upon the vessels then was obtained, so that about one-third of the suspension was illuminated. Under these conditions, the illumination intensity was in general such that its effects were negligible compared to those of the actinic illumination. Quartz optics were applied to enable measurements in the ultra violet. In order to minimize light losses due to scattering a photomultiplier (RCA 5819 in the blue and near u.v. region) with a large photosensitive surface (5 cm diameter) was placed at a distance of 6 cm above the vessels to catch a large fraction of the scattered radiation.

A high stability was obtained in the first place by means of the compensation of the light beams by each other. Consequently, effects which influence both beams equally in principle do not cause a deflection of the recorder. This applied for such effects as variations of the light emission of the lamp, and of the sensitivity of the multiplier, amplifier and recorder. Also mechanical disturbances which affect both beams equally are compensated in this way. The optics of both light paths were made identical as far as possible. The photomultiplier was placed symmetrically above the vessels at the intersection of the two beams.

Much care was taken to avoid mechanical tremblings and vibrations of the optics of the apparatus. The whole set-up was placed upon a rigid aluminium table, the top of which was equipped with reinforcement ribs, and, to avoid bending, rested upon three legs, standing on rubber blocks. All optical parts, including photomultiplier, motor and lamp, were fastened rigidly upon the table.

Although the effect of settling of the suspension, which occurs with many organisms, is in first approximation compensated in a split-beam apparatus, some larger microorganisms may settle so rapidly, that troublesome effects occur when the vessels are mounted vertically, as is usual in spectrophotometry. For this reason an optical arrangement was chosen which permitted a horizontal mounting of the cuvettes. This had the advantage that even extensive settling had only little effect upon the absorbancy and the scattering of the suspension, and made also possible measurements with completely settled samples. Open, completely filled vessels of 1 mm thickness could be mounted horizontally without any precaution, because the surface tension of the suspension prevented leakage. The 1 cm vessels used were glass-stoppered.

The light attenuators at_1 and at_2 were constructed to give an approximately homogeneous weakening of the measuring beams. One of these, at_2, consisted of a rectangular frame, to which were attached at equal distances nearly vertical metal wires of 0.3 mm thickness. Turning at_2 around a vertical axis resulted in the gradual introduction (or withdrawal) of more wires into the beam, so that its intensity could be continuously varied. Fine-adjustment was accomplished by means of a micrometer

screw. The transmittance of at_2 ranged from about 90 to about 80%. The design of at_1 was similar, but instead of wires a series of thin parallel lamellae was used. at_1 provided an effective transmittance range of about 90 to about 20%; in this way a coarse adjustment was made possible.

In the u.v. region, the sensitivity of the apparatus was found to be determined by the noise of the photomultiplier due to photo-electrons and thus to be dependent on the transmittancy of the sample in the vessel. At 340 mμ the sensitivity with highly transparent samples was about 3×10^{-5} absorbancy units. At longer wavelengths where more light was available, a sensitivity of 1 to 2×10^{-5} absorbancy units was obtainable, also at wavelengths where the noise of the photo-electrons would have permitted a better resolution. The reason of this instability is uncertain yet.

Vessel V_2 could be illuminated with actinic light as shown in Fig. 2.4.B. The slightly enlarged image of a rectangular stop, placed in the slide transport of an Aldis Star 500 5 x 5 cm slide projector, was projected by means of lens L_4 upon the cuvette. The light was filtered by a 2 cm layer of water (F_2) to absorb heat radiation, and by the filter combination F_3 to render the desired spectral composition. Part of the light was reflected by means of the glass plate G; a Lange silicon photocell, situated at the place of the image served to measure the intensity of irradiation. The figure shows the way of illumination of a 1 mm absorption vessel. The light reflected by mirror M_5 fell upon the surface of the cuvette with an angle of incidence of 57° from the vertical. The whole surface of the vessel (50 x 10 mm) was illuminated; the intensity was varied by means of a variable transformer connected to the projection lamp. Vessels of 1 cm light path were illuminated by means of the same optics. The set-up, consisting of projector, lens L_4, filters, glass plate and silicon cell, was constructed as one unit, so that its position could easily be changed in such a way, that the image of the stop was projected directly, without passing mirror M_5, upon the side wall of the vessel.

The silicon photocell was calibrated, for each filter combination F_3, with a calibrated Kipp thermopile situated at the place of the vessel.

The light of the photosynthetic active illumination was not modulated at 50 Hz: so light, scattered by the suspension and reaching the multiplier does not, in principle, cause a recorder deflection. Upon shutting on and off the light, however, serious disturbances occurred. The illumination may also increase the noise and change the sensitivity of the photomultiplier. For this reason complementary filter combinations F_1 and F_3 were chosen for each experiment. Filter F_1 also served to reduce stray light and, if necessary, higher order spectra of the monochromator.

For measurements around 340 mμ, F_1 was a Schott UG 11 filter of 4 mm thickness. In the blue region F_1 consisted of Schott AL, or Balzers B40 or K-type interference filters, com-

bined with blue glass filters, such as Schott BG 12, BG 23 and BG 38 to cut off red and infra red radiation, or of these BG filters only. F_3, filtering the actinic illumination, consisted of a combination of Schott RG or OG "cut-off" filters, usually in combination with interference filters, and if possible with heat-absorbing and Balzers Calflex heat-reflecting filters.

In order to be able to illuminate the suspension simultaneously with light of two different wavelengths and to measure action spectra of absorption changes the optics for illumination have been modified later in such a way that, except by means of an Aldis projector, vessel V_2 could also be illuminated by means of a second Bausch and Lomb monochromator. This monochromator was equipped with an Osram 900 W Xenon arc XBO 1001; a reduced image of its grating (10 x 10 mm) was projected upon V_2. By means of a half-transparent mirror, V_2 could be simultaneously illuminated by the Aldis projector. Both light beams fell on the vessel with an angle deviating 67° from the vertical, so that with a 1 cm vessel both the upper and the side walls were illuminated. The monochromator illuminated only 10 mm of the length of the vessel; for this reason the height of the exit slit of the other monochromator then was reduced to 5 mm. The light intensity was measured by means of a calibrated Lange silicon photocell, which was illuminated by turning away a mirror placed in the light beam.

The increase of absorption in the ultra violet occurring upon reduction of added NADP or NAD by an illuminated chloroplast suspension proceeds for a much longer time than that observed in intact cells, because the accumulation of $NADPH_2$ or $NADH_2$ usually goes on for several minutes. These absorption measure-

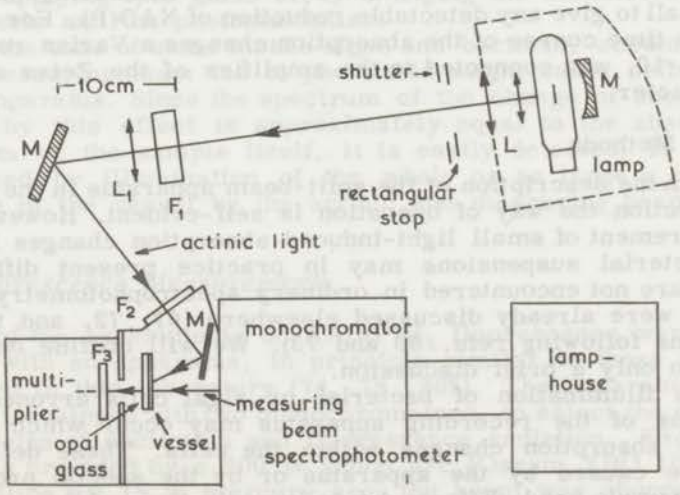

Fig. 2.6 Attachment used for measurement of the light-induced reduction of NADP and NAD by a preparation of chloroplasts. The chloroplast suspension was contained in the absorption vessel. M are mirrors, L are lenses and F are filters.

ments were carried out in the Zeiss PMQ II spectrophotometer, equipped with an attachment for providing actinic illumination as illustrated in Fig. 2.6. A rectangular diaphragm placed in the slide transport of the Aldis projector was imaged by means of lens L_1 and two mirrors on the 1 mm cuvette. The light was filtered by a 2 cm layer of water (F_1) and a combination of absorption and interference filters. The lid of the vessel compartment was replaced by one of modified design, to which the filter holder (F_2) and the second mirror were attached. The actinic beam was incident from a direction, deviating 26.5° from the measuring beam, the latter being perpendicular to the surface of the vessel. Behind the absorption vessel a piece of opal glass was placed to minimize the effect of light scattering. The change in absorption in the cuvette, which was in contact with the opal glass, was measured at 350 mμ. The whole surface of the cuvette was illuminated by the actinic beam. The intensity was measured by means of a small calibrated Lange silicon photocell, placed in a second section of the movable cuvette holder.

In the Zeiss spectrophotometer the measuring beam is modulated with a frequency of 50 Hz and the detecting apparatus is sensitive only to light which is modulated with this frequency. To prevent, however, spurious effects caused by the actinic light, a Schott UV-IL interference filter with maximum transmission at 348.5 mμ and a half-width of 8 mμ was placed in front of this multiplier.

In order to get a sufficiently high sensitivity, the apparatus was operated with maximum slit-width. The half-width of the measuring beam was thus determined by the half-width of the interference filter. The intensity of the measuring beam was too small to give any detectable reduction of NAD(P). For recording the time course of the absorption changes a Varian recorder, type G-10, was connected to the amplifier of the Zeiss spectrophotometer.

2.4.2 Methods

From the description of the split-beam apparatus in the preceding section the way of operation is self-evident. However, the measurement of small light-induced absorption changes in algal or bacterial suspensions may in practice present difficulties which are not encountered in ordinary spectrophotometry. Some points were already discussed elsewhere (67, 72, and the discussions following refs. 60 and 73). We will confine ourselves here to only a brief discussion.

Upon illumination of bacterial or algal cells erroneous deflections of the recording apparatus may occur which are not due to absorption changes within the cells. These deflections may be caused by the apparatus or by the special properties of the sample used. Effects due to imperfections of the apparatus were discussed already. The apparatus may be checked by a control experiment with killed or inhibited organisms.

Among the spurious effects caused by the sample may be mentioned:

1. The actinic light may excite fluorescence. In practice this effect only presents difficulties (such as an increase of noise or transients upon onset or cessation of actinic illumination) with measurements in the region of bacteriochlorophyll, chlorophyll *a* or phycocyanin fluorescence, since in other spectral regions it can be suppressed or minimized by means of filters. These effects (except when caused by a non-linearity of the response of the photomultiplier) can be partly checked by shutting off the measuring beams and can be diminished by adding a narrow band pass interference filter to F_1 (Fig. 2.4.B).

2. The effect of a change in the fluorescence excited by the measuring beams upon adding actinic light may be more troublesome, because this fluorescence light is modulated (see discussion following ref. 60). Control experiments are in principle possible by changing the geometry of the optics. The effect may be more easily checked by adding a filter to F_1 (Fig. 2.4.B), which cuts off the measuring beam but transmits part of the fluorescence, or by adding a narrow band pass filter to F_1 in which way the effect is diminished.

3. With a dense suspension a relatively large part of the light may not pass the entire length of the suspension, but reach the multiplier by way of the side walls of the vessels (165). The effect can be checked by dilution of the suspension and minimized by the use of appropriate light stops if necessary.

4. Since the extent of scattering changes, induced by actinic illumination, is dependent upon the geometry of the optics, it may in principle be checked by a modification of this geometry, e.g. by applying opal glass or by changing the distance between the vessels and the photomultiplier.

5. In the case of some motile algae and bacteria, accumulation in the measuring beam due to phototaxis might cause deflections of the apparatus. Since the spectrum of the change of absorption caused by this effect is approximately equal to the absorption spectrum of the sample itself, it is easily detected. It can be minimized by illumination of the whole or as large a part as possible of the vessel by the actinic and measuring beams.

2.5 *Fluorescence Measurements*

Part of the experiments on $NAD(P)H_2$ fluorescence were performed with an apparatus, in principle similar to those earlier employed in this laboratory (74, 75, 205). The apparatus was, however, equipped with two monochromators, to select the desired wavelengths of excitation and fluorescence radiation. Excitation light was provided by a 900 W xenon arc (Osram XBO 1001) or by a Philips HP 75 W mercury arc; the beam was chopped at a frequency of 50 Hz by means of a rotating sectioned disc, driven by a synchronous motor. Actinic illumination was provided by

an Aldis slide projector without projection lens and heat filter, in a similar way as described in the previous section. Suitable light filters served to minimize the effects of stray light, and of scattered excitation and actinic light. The electronics were the same as of the absorption difference spectrophotometer (Fig. 2.5). However, this apparatus, which enabled the measurement of fluorescence action and emission spectra, had the disadvantage that only a small area of the 1 mm quartz vessel containing the algal or bacterial suspension was illuminated by the excitation beam, because the emitted fluorescence had to be fed into the second monochromator. For this reason, the light of the xenon or mercury arc had to be weakened, in order to get a permissable intensity of illumination on the suspension. In experiments where a higher sensitivity was necessary, and when no fluorescence emission spectrum had to be measured, a different set-up was used.

Fig. 2.7.A shows an optical arrangement for measuring

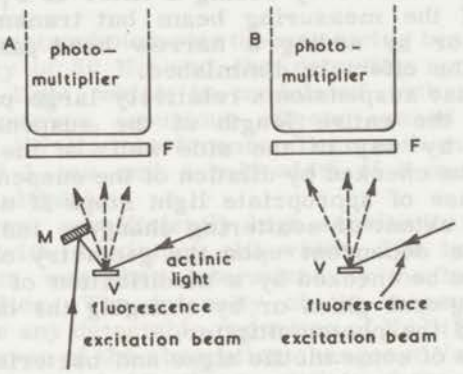

Fig. 2.7 Attachments for measuring fluorescence by means of the absorption difference spectrophotometer of Fig. 2.4. In both set-ups the fluorescence excitation beam was chopped at 50 Hz.

$NAD(P)H_2$ fluorescence, obtained by a slight modification of the optics of the absorption difference spectrophotometer described in the previous section (cf. Fig. 2.4). Fluorescence was excited by means of the left of the two beams of the apparatus, which was reflected by means of a small mirror on vessel V_2. The other beam was shut off. Photosynthetic illumination was effected by means of a second monochromator or an Aldis projector as described already. Vessel V_2 was a 1 mm quartz absorption cuvette. The fluorescence was filtered by a combination consisting of Schott FG 10, 1 mm, WG 1, 2 mm and BG 23, 2 mm, Balzers Filtraflex K2 and Schott BG 23, 5 mm, which transmitted 26% at 450 mμ and less than 0.1% below 400 and above 530 mμ.

A Schott UG 11 filter of 4 mm thickness placed behind lens L_3 (Fig. 2.4.A) served to minimize false light. A GEC ME/D

250 W high pressure mercury arc, selected for stability, or an AEI 500 W a.c. or Osram 1001 900 W d.c. xenon arc placed in front of the monochromator, which was set at 334 or 340 mμ, provided the exciting radiation. The electronic part of the apparatus was not modified: the photocurrent of the RCA 5819 photomultiplier was measured as described in the previous section. In some experiments the recorder deflection was partly balanced by a variable voltage to increase the measuring range. Because a much larger part of the suspension is irradiated by the exciting beam than in the first apparatus a larger energy flux could be applied for a given intensity. The photomultiplier, moreover, caught a larger fraction of the emitted fluorescence, so that a higher sensitivity was obtained. Under the experimental conditions employed, photosynthetic effects caused by the ultra violet irradiation were found to be negligible compared to those caused by the photosynthetic illumination. A disadvantage of the set-up was that the multiplier received a relatively large fraction of the false fluorescence emitted by the filter F and excited by scattered excitation light, because the filter was situated close to the photomultiplier. Since only fluorescence changes of the suspension were recorded, this was no serious objection. The filter combination was carefully selected in order to minimize this false fluorescence.

Fig. 2.7. B shows the arrangement used for the measurement of the action spectrum of bacteriochlorophyll fluorescence. The second monochromator, which in other experiments had been used in order to obtain monochromatic photosynthetic illumination, now provided light for exciting fluorescence. The set-up was not modified, except for a light chopper which was placed in the exciting beam. The number of incident quanta per sec was measured by means of the silicon cell mentioned in the previous section. The light source was a xenon arc. The light from the monochromator was filtered by means of suitable Balzers B 40 interference filters to reduce false light. The fluorescence was filtered by means of an infra red filter XRX 40 of Polaroid Corporation and a special Kodak filter transmitting above about 900 mμ, and was measured by means of a Dumont 6911 photomultiplier.

2.6 *Photosynthesis Measurements*

The rate of oxygen production was measured by means of an oxygen polarograph. This polarograph was equipped with a large, flat, stationary platinum electrode, following the principle applied by Blinks and Skow (38). Electrodes of this type have originally been constructed for the measurement of photosynthetic oxygen production of thalli of green, red and brown algae (see also ref. 123). Although such an electrode can also be used for the measurement of photosynthesis of unicellular algae (67), we have used a modified design (cf. ref. 194), better adapted to this purpose.

The electrode, a horizontal square plate of bright platinum of 5 x 5 mm^2, was embedded in a perspex block, in such a way that its upper surface, which was in contact with the solution, was 0.7 mm lower than the surface of the perspex. So a hole of 0.7 mm deep was formed, the bottom of which was formed by the electrode. A drop of an algal suspension of suitable concentration (0.5 - 2%) was brought on the electrode, and covered by a cellophane membrane (Kaller dialysis tube). The electrode was mounted horizontally in a vessel of about 30 ml capacity, which was subsequently filled with growth medium, saturated with air and 5% CO_2. The membrane, which separated the electrode compartment from the medium in the vessel, permitted electrical and chemical transport (except for large molecules) between the two compartments. The electrolyte in the polarograph vessel was connected to a saturated calomel electrode by means of an agar bridge, saturated with KCl.

When given a suitable negative potential, a bright platinum electrode like the one described here carries an electrical current, which, unless interfering substances are present, is proportional to the amount of oxygen per sec reduced at the electrode. Since the latter is determined by the rate of diffusion of oxygen to the electrode and this rate is proportional to the oxygen concentration, the current, at a given temperature, is proportional to the oxygen concentration of the solution in which the electrode is immersed.

Photosynthetic oxygen production was now measured as follows: A potential of -0.6 V was applied to the platinum electrode with respect to the calomel electrode. After 1-2 h, when the algae had been settled in a thin layer upon the electrode and the diffusion rate had become stationary, the electrical current of the polarograph reached a constant value. The polarograph current gave rise to a voltage of 1-2 mV over a resistance of 1000 Ω, which was incorporated in the circuit. This voltage was approximately compensated by means of a potentiometer and battery. The remaining signal was amplified by means of a Hewlett-Packard d.c. μV meter model 425A and recorded by means of a Varian recorder type G-10.

Experimentally it was found that, for not too high light intensities, the difference between the steady state levels of the light and dark current of the polarograph was proportional to the light intensity for several algal species tested, which indicated a linear relationship between photosynthesis and polarograph current.

Photosynthetic illumination could be effected by means of a Bausch and Lomb grating monochromator, equipped with a 6 V 17 A tungsten ribbon filament lamp. The grating of the monochromator was projected upon the electrode, so that the slit-width could be varied without changing the illuminated area. The electrode could also be illuminated simultaneously by means of an Aldis Star 500 slide projector, equipped with suitable interference and absorption filters. Glass plates, placed in the

two light beams, reflected part of the light upon RCA 925 vacuum photocells to measure the incident light intensity.

Part of the light transmitted by the layer of algae was reflected by the platinum electrode, and again partly absorbed by the algae. Since the platinum reflectance varies with wavelength, a correction for the effect had to be made, when comparing the activity of light of different wavelengths, also for thin algal layers of low absorptancy. For this correction reflectance factors were used given by Haxo and Blinks (123), who reported a linear variation between 57.5% at 400 and 73.5% at 740 mμ.

For filamentous algae an electrode was used, the surface of which was at the same level as that of the perspex block. The algae were pressed against the platinum surface by means of the dialysis tube.

CHAPTER III

PHOTOSYNTHETIC BACTERIA

3.1 *Introduction*

In this chapter the results are reported of experiments on NAD(P) reduction in intact cells of the non-sulphur bacteria Rhodospirillum rubrum and Rhodopseudomonas spheroides. Duysens and Sweep (75) showed that intact cells of R. rubrum and Chromatium, strain D, emit a blue fluorescence upon excitation with ultra violet radiation. When the cells were suspended in a suitable medium, the fluorescence increased upon photosynthetically active infra red illumination. The emission (75, 204) and excitation (203, 205) spectra of the increase of blue fluorescence were found to be similar to those of enzyme-bound $NADH_2$. Since the infra red light itself was not able to excite $NAD(P)H_2$ fluorescence these experiments indicated that the fluorescence increase was due to an increase of the intracellular concentration of $NADPH_2$ or $NADH_2$, caused by a light-induced reduction of coenzyme. In these experiments and those to be reported in this and the following chapter, the intensity of ultra violet illumination was too low to cause a measurable reduction of NAD(P). In Chromatium, the quantum requirement for NAD(P) reduction was found to be between about 1.5 and 6 quanta per reduced equivalent (76, 78). The uncertainty in this figure was caused by the fact that the fluorescence yield of $NAD(P)H_2$ *in vivo* was only known approximately (cf. ref. 224).

The results of the experiments to be reported here indicate amongst others things, that in bacteria which had been kept in organic media in darkness during a few min, a relatively large pool of NAD or NADP was reduced at a high rate during the first sec of illumination, but that during illumination both the rate of reduction and the amount of reduced coenzyme in the cell declined, usually after less than 1 min, and that the rate of coenzyme reduction fell to a much lower value after a few min of illumination. This suggests that under the conditions of these experiments the reduction of NAD(P) in the light is quantitatively not very important during steady state conditions.

The similarity of the action spectra of NAD(P) reduction and bacteriochlorophyll fluorescence in Rhodopseudomonas was consistent with the hypothesis that only one pigment system is present in photosynthetic bacteria.

The implications of these findings, together with evidence reported by others, will be discussed in § 3.4.

3.2 *Materials and Methods*

Rhodospirillum rubrum (von Esmarch) Molisch, strains 1 and 4, was grown either in peptone or in synthetic media.

The bacteria in peptone medium, which contained 1% peptone (Difco) and 0.5% NaCl, were grown, either in sterile, completely filled, glass stoppered flasks, or with constant bubbling of N_2 (see § 2.1), at 25-30°C and at a light intensity of about 2000 lux, supplied by incandescent lamps.

The bacteria in synthetic media were grown at 30°C and at about 4000 lux. These media were basically the same as that described by Cohen-Bazire *et al.* (59) except for a higher concentration of NaCl, necessary for growing strain 4, and different concentrations of organic substrates. Stock solution No. 2 of the above authors was replaced by a solution containing 190 g of NaCl and 53.5 g of NH_4Cl per 1 and adjusted to pH 6.8 with NaOH. Before autoclaving 1 g of L-glutamic acid and either 1.7 g of sodium acetate, 1.5 g of sodium butyrate, 1.5 g of DL-malic acid or 1.5 g of sodium succinate were added to 1 l of the medium, and the pH was brought to 7.0 with NaOH. These media will be referred to as "acetate", "butyrate", "malate" and "succinate" medium. The cultures were routinely gassed with a mixture of 95% N_2 and 5% CO_2. Strain 1 also grew well in malate and acetate medium gassed with N_2 only.

Rhodopseudomonas spheroides van Niel was grown in malate medium or in the medium of Cohen-Bazire and coworkers, unmodified except for a higher concentration of NaCl (3.8 g/l). The casamino acids were replaced by L-glutamic acid and sodium acetate as indicated (59). The culture was gassed with a mixture of 95% N_2 and 5% CO_2.

Unless otherwise stated, the bacteria were harvested by centrifugation and resuspended, usually in fresh growth medium. The suspension was gassed for at least 45 min, normally with the mixture of N_2 and CO_2, and before measurement transferred to 1 mm or 1 cm quartz absorption vessels. The experiments with Rhodospirillum rubrum were done with strain 1 unless otherwise indicated.

HOQNO was a kind gift of Dr. J. W. Lightbown, London.

The absorptancy of the bacterial suspensions was measured and corrected for scattering as described in § 2.3. Changes in absorbancy resulting from actinic illumination were measured by means of the apparatus described in § 2.4. In some experiments $NAD(P)H_2$ fluorescence was measured by means of the apparatus equipped with two monochromators (§ 2.5). The other experiments were done with the set-up of Fig. 2.7.A. With a few exceptions, the actinic illumination was filtered by means of Balzers B40 interference filters (10-15 mμ half-width) and a Schott RG 9 filter of 4 mm thickness. Bacteriochlorophyll fluorescence was measured by means of the set-up illustrated in Fig. 2.7.B.

Unless otherwise stated the experiments were performed at

room temperature (about 22°C).

Since the absorption changes brought about by $NAD(P)H_2$ around 340 mμ in Rhodospirillum rubrum upon illumination are relatively large, the effects of scattering flattening (§ 2.2) and of deviations of Beer's law could be checked directly at this wavelength.

Using a 1 mm vessel, we found, upon illumination with strong light of saturating intensity, the maximum change of absorption at 340 mμ of a suspension of butyrate-grown Rhodospirillum to be linear with the concentration up to a bacterial concentration of 4%, the highest applied in our experiments. This indicates that within the experimental error of a few percent, Beer's law applied for these suspensions.

The effect of scattering flattening was measured by comparing the absorbancy difference readings with and without opal glass, as discussed in § 2.3. The results, for suspensions of butyrate and malate-grown Rhodospirillum, gave no measurable difference indicating that the effect of scattering flattening was negligible for the geometry used. This result also demonstrates that the recorder deflections were only due to absorption changes, since scattering changes (§ 2.4) would have been much smaller with than without opal glass.

A rough estimate of the amount of particle flattening was obtained by measuring the absorbancy (with opal glass) of a suspension of known concentration of butyrate-grown bacteria at 340 mμ and counting the number of cells per unit volume. According to equation (2.7) for homogeneously colored spherical cells the measured values would have given an average transmittancy $T'_{p\ av}(\gamma)$ of 0.94 and a flattening (Fig. 2.1) of 0.93. Since the bacteria are spirilla instead of spheres, the average transmittancy was probably nearer to unity, and consequently the effect of flattening probably smaller.

Since all three effects discussed apparently were small for Rhodospirillum rubrum, no corrections were applied in the calculation of the quantum requirements for NAD(P) reduction reported in this chapter.

3.3 *Results and Interpretation*

3.3.1 The identification of $NAD(P)H_2$

As mentioned in § 3.1, experiments of Duysens, Olson and coworkers (75, 203, 205) indicated that the increase of blue fluorescence upon illumination of Rhodospirillum rubrum is due to a light-induced reduction of NAD or NADP. We found, that the kinetics and the emission spectrum of the blue fluorescence changes in Rhodospirillum rubrum strain 4 were the same for excitation with radiation of 280 and 340 mμ. This gives further evidence that the fluorescence changes are caused by (bound) $NAD(P)H_2$. On basis of fluorescence measurements it cannot

be decided whether NAD or NADP is reduced: in agreement with findings of Lowry *et al.* (186) we found the fluorescence spectrum and fluorescence yield of solutions of $NADPH_2$ and $NADH_2$ of the same concentration to be identical within the experimental error of a few percent.

Although measurement of fluorescence provides a sensitive and specific way of studying the reduction of NAD(P) *in vivo*, the method is less satisfactory for quantitative determination, because the fluorescence yield of $NAD(P)H_2$ bound to cell constituents differs by an unknown factor from that of free $NAD(P)H_2$ (cf.

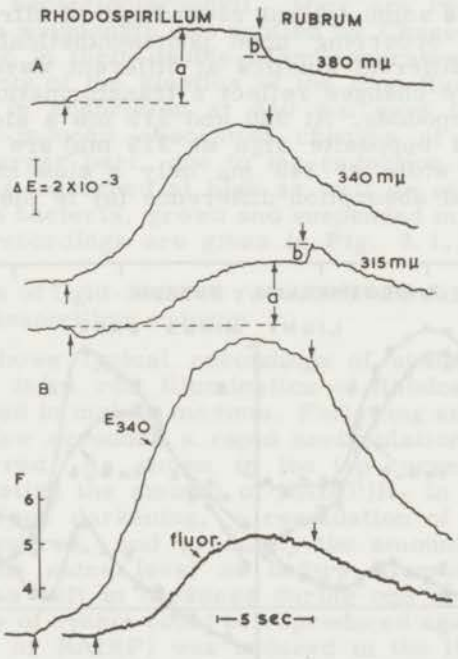

Fig. 3.1A Recordings of kinetics of absorption changes in the near ultra violet at 3 different wavelengths upon illumination of 2 days old malate-grown Rhodospirillum rubrum, strain 1. An upward moving trace indicates an increase of absorption. The bacteria were examined in fresh growth medium, in a concentration of 2% wet cells in a 1 mm vessel. Actinic illumination was provided by a band around 838 mμ of an intensity of 6.4×10^{-9} einstein/(sec cm^2). (One einstein equals N light quanta, where N is Avogadro's number; 10^{-9} einstein equals 1.39×10^3 erg at 860 mμ). Onset of illumination is indicated by an upward pointing arrow and darkening by a downward pointing one.

Fig. 3.1B A comparison of the kinetics of changes of absorbancy and fluorescence of a 4% suspension upon illumination. The fluorescence, expressed in arbitrary units, was excited by light of 334 mμ and measured at 450 mμ. The other conditions were the same as in Fig. 3. 1A.

ref. 224). Measurement of absorption has the advantage that the absorption spectrum of $NADH_2$ or $NADPH_2$ in the near ultra violet is generally less affected by binding to an enzyme than the fluorescence spectrum: the wavelength of maximum absorption may be somewhat shifted, but the height of the absorption maximum is little changed (224). Furthermore, the shape of the fluorescence excitation spectrum (205) indicates that in Rhodospirillum no substantial shift of the absorption maximum occurs. However, absorption measurements are less specific, because reactions of cytochromes and bacteriochlorophyll give also rise to absorption changes in the ultra violet.

Fig. 3.1A shows some typical recordings of ultra violet absorption changes occurring upon photosynthetically active illumination. The different kinetics at different wavelengths show clearly that these changes reflect a transformation of at least two different compounds. At 380 and 315 mμ a slow phase and a fast one (*b*, of oppposite sign at 315 mμ) are distinguished upon darkening, while at 340 mμ only a slow change is discernible. The total absorption difference (*a*) is the sum of both components.

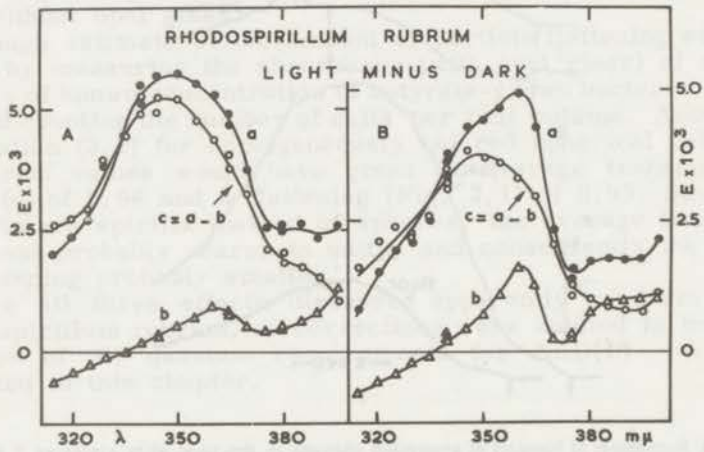

Fig. 3.2A Difference spectra of the maximum (a) and rapid (b) change in absorbancy of a suspension of malate-grown Rhodospirillum rubrum. The change c = a - b may be taken to be the magnitude of the slow change. The absorption changes and experimental conditions are the same as those in Fig. 3.1A. Further details are given in the text.

Fig. 3.2B The same difference spectra, but obtained with a 2% suspension of 2 days old Rhodospirillum rubrum, grown in peptone and examined in a medium containing butyrate and phosphate, in the presence of N_2 and 5% CO_2. The wavelength of actinic illumination was 860 mμ, the intensity was 6.2×10^{-9} einstein/(sec cm^2).

The difference spectrum of the maximum deflection in the light minus the steady state in the dark (*a*) and of the fast change

(*b*) are given in Fig. 3.2.A. The approximate spectrum of the slow absorption change, curve *c*, is obtained by taking the difference of curves *a* and *b*. Fig. 3.2.B shows similar difference spectra measured under different experimental conditions. It can be seen that the spectra marked *c*, of Fig. 3.2.A and B are approximately proportional to the absorption spectra of $NADH_2$ or $NADPH_2$. The shape of the spectra indicates that, at 330-340 mμ, interfering absorption changes caused by other substances are small. The fast absorption changes at other wavelengths are probably caused by light-induced oxidations of cytochromes and bacterioclorophyll. They are relatively small at 370 mμ, which wavelength was applied by Chance and Olson (52) as a reference in their double-beam apparatus.

The similarity of the kinetics of the blue fluorescence and of the increase in absorption at 340 mμ gave further indication that the light-induced absorption changes at 340 mμ are, at least for the larger part, due to the reduction of NAD(P). This similarity was observed at high as well as at low light intensities and with bacteria, grown and suspended in different media. Two typical recordings are given in Fig. 3.1.B.

3.3.2 Kinetics of light-induced reduction and oxidation of NAD(P) in Rhodospirillum rubrum

Fig. 3.3 shows typical recordings of absorption changes at 340 mμ upon infra red illumination of Rhodospirillum, grown and resuspended in malate medium. Following an induction period of one or a few seconds, a rapid accumulation of reduced coenzyme occurred. As shown in the top curve, after about 10 sec of illumination the amount of $NAD(P)H_2$ in the cells slowly diminished. Upon darkening, a reoxidation of the accumulated $NAD(P)H_2$ occurred, and gradually the amount dropped to approximately the same level as before illumination. After the suspension was left in darkness during one or a few min, the same sequence of events could be reproduced again. A relatively large amount of NAD(P) was reduced in the light: after a few seconds of illumination of sufficiently high intensity the concentration of photoreduced coenzyme in the cells was roughly one-tenth of that of bacteriochlorophyll.

From the data of Table 3.1 it appears that, after illumination periods ranging from 6 to 30 sec, the rate of reoxidation of $NAD(P)H_2$ is for a given sample of Rhodospirillum approximately proportional to the amount of light-reduced coenzyme present in the cells, and independent of the other experimental parameters, such as the intensity and duration of illumination. Measurement of the oxidation rate at different temperatures indicated a relatively small temperature dependence of the reaction corresponding to an activation energy of about 3000 gcal/gmol in the range of 1° to 37°C. The data suggest that $NAD(P)H_2$ is oxidized in a dark reaction by substances present at an approximately constant concentration (perhaps at a large amount).

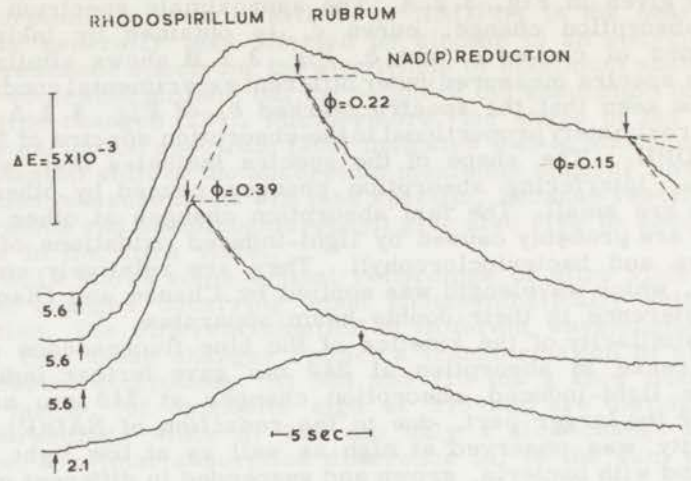

Fig. 3.3 Kinetics of NAD(P)-induced absorption changes at 340 mμ in Rhodospirillum rubrum upon illumination of different duration and intensity. The bacteria, 2 days old, were grown and examined in malate medium in a 1 mm vessel, concentration: 4% wet cells. The wavelength of actinic light was 860 mμ, the intensity was 2.1 for the lowest curve and 5.6×10^{-9} einstein/(sec cm^2) for the other curves. An upward moving trace indicates an increase of absorbancy and a reduction of NAD(P). ϕ indicates the quantum efficiency of the reduction, calculated as described in the text from the difference between the slopes of the curves before and after darkening.

Since the rate of the reoxidation can be measured by shutting off the light, the total rate of photoreduction of NAD(P) can be obtained from the difference between the slopes of the curve before and after darkening (see Fig. 3.3). The three top recordings show that this rate is appreciably higher after 5.7 sec of illumination (third recording) than after 11.7 or 28.5 sec. The small "overshoot" which is observed in the third recording upon darkening is left out of consideration here and will be discussed below.

TABLE 3.1

Dark oxidation of $NAD(P)H_2$ after illumination of Rhodospirillum rubrum.

Sample No.	Light intensity	Duration of illumination (sec)	Half-time for $NAD(P)H_2$ oxidation upon darkening (sec)		
			first	second	third
1	10.3	12	5.0	4.8	
1	10.3	27	4.5	4.6	
1	5.6	6	4.2	5.5	
1	2.1	16	4.7	5.0	
1	2.1	30	4.1	4.8	
2	10.3	13	3.6	3.6	3.6
2	5.6	11	3.2	3.5	3.8
3	5.8	9	3.0	3.3	4.0
3	2.1	22	3.8	4.7	

The measurements were done with malate-grown Rhodospirillum rubrum. Samples Nos. 1 and 3 were taken from two different 2 days old cultures, No. 2 from a 1 day old culture. The experimental conditions were the same as in Fig. 3.1.A.

The time required for lowering the concentration of photoreduced $NAD(P)H_2$ to half the value of that upon cessation of actinic illumination is given as the first half-time of the reaction. The second and third half-time indicate the time required to lower the concentration with a factor 2 again.

The wavelength of actinic illumination was 860 mμ for sample Nos. 1 and 2, and 838 mμ for No. 3. The light intensities are given in 10^{-9} einstein/(sec cm^2).

3.3.3 Quantum requirement and kinetics of NAD(P) reduction in different media

The quantum efficiency of the reduction of NAD(P) was measured as the number of equivalents of $NAD(P)H_2$ (one equivalent is half a molecule) produced per sec in a given volume of the suspension, divided by the number of light quanta per sec absorbed in the same volume. The number of equivalents of reduced coenzyme formed by the photoreduction was calculated from the "total" rate of the reaction, which was calculated from the measured differences between the rates of absorbancy changes before and after darkening as described in the previous section. A specific absorption coefficient of 6.22 cm^{-1} mM^{-1} at 340 mμ (139) was applied for $NAD(P)H_2$. The number of einsteins was calculated from the intensity of the actinic light and from the absorptancy of the suspension. Corrections were applied for the obliqueness of the incident beam, for reflections at the cuvette walls and for an inhomogeneity of the actinic beam.

Since the actinic light fell upon the upper wall of the vessel with a deviation of 57° from the vertical (§ 2.4.1) the intensity of the incident light was multiplied by a factor of 0.55 (cos 57°) to ob-

tain the energy flux for a surface of 1 cm^2 of the vessel. The value obtained was multiplied by 0.93, because of reflection at the wall of the vessel.

The measured absorbancy of the suspension was multiplied by 1.26, because the actinic light passed the vessel obliquely, and by 1.07 because of reflection at the lower wall of the vessel. The corrected absorbancy was converted to the corresponding absorptancy. Since the absorptancy of the suspensions was higher than 0.85, the above corrections had only a relatively small influence. The inhomogeneity of the actinic beam was checked by means of a small silicon cell, placed at different positions in the vessel holder; the inhomogeneity did not exceed 10% of the average value in the region where the measuring beam passed.

Fig. 3.4 gives the quantum efficiency of coenzyme reduction obtained at three different light intensities, as a function of the

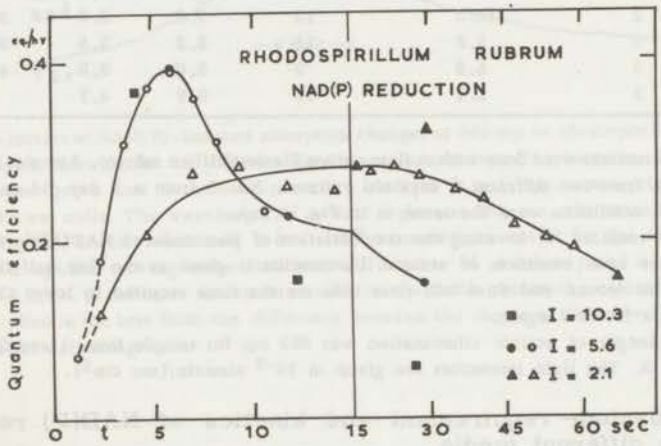

Fig. 3.4 The quantum efficiency of NAD(P) reduction in malate-grown Rhodospirillum rubrum as a function of the time of illumination, measured at different light intensities (expressed in 10^{-9} einstein/(sec cm^2)). The experimental conditions were the same as in Fig. 3.3. Solid squares, circles and triangles were obtained by subtracting the rates obtained from the slopes of the recording immediately before and after darkening as described in the text. Open circles and triangles were calculated for two recordings from the measured slope during illumination at the time indicated and from the rate constant of the dark oxidation of NAD(P)H_2.

time of illumination of the same suspension as that of Fig. 3.3.

The light-induced absorption and fluorescence changes of bacteria grown and suspended in acetate, butyrate or succinate medium were partly similar to those in bacteria grown in malate (Fig. 3.5). Like in malate-grown bacteria, the rate of coenzyme reduction and the amount of reduced NAD(P) reached a maximum after a few sec of illumination and afterwards gradually dropped to much lower values. However, for butyrate-grown bacteria a

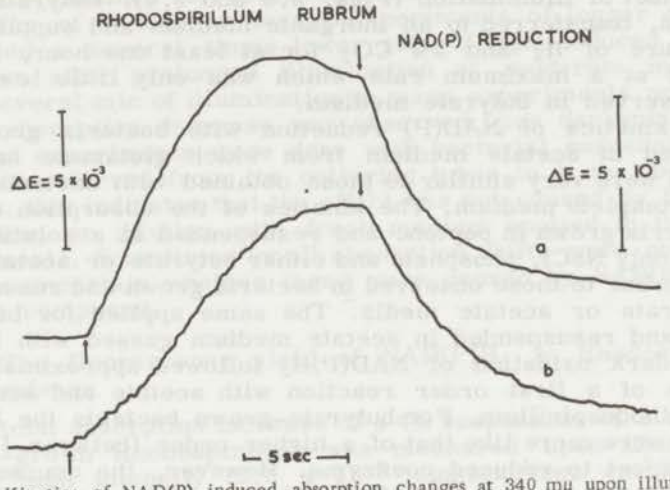

Fig. 3.5 Kinetics of NAD(P)-induced absorption changes at 340 mμ upon illumination in 2 days old Rhodospirillum rubrum, grown and resuspended in butyrate medium (curve a) and in succinate medium. The light intensity was 3.5×10^{-9} einstein/(sec cm^2). The other conditions were the same as in Fig. 3.3. The scale for curve (a) is given at the left, that for curve (b) at the right-hand side of the figure.

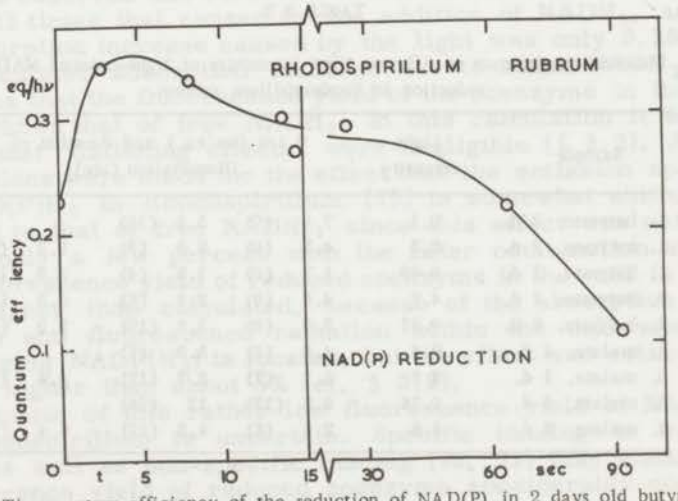

Fig. 3.6 The quantum efficiency of the reduction of NAD(P) in 2 days old butyrate-grown Rhodospirillum rubrum, measured as a function of the duration of illumination at an intensity of 5.3×10^{-9} einstein/(sec cm^2). The point at 0 sec was calculated from the initial increase of absorbancy at 340 mμ upon illumination; the other points were calculated in the same way as the solid squares in Fig. 3.4. The experimental conditions were the same as in Fig. 3.5, curve (a).

relatively rapid reduction was observed already immediately after onset of illumination (Figs. 3.5 and 3.6). Butyrate-grown bacteria, transferred to an inorganic medium and supplied with a mixture of H_2 and 5% CO_2 for at least one hour, reduced NAD(P) at a maximum rate which was only little lower than that observed in butyrate medium.

The kinetics of NAD(P) reduction with bacteria grown and examined in acetate medium from which glutamate had been omitted were very similar to those obtained with bacteria grown in the complete medium. The kinetics of the absorption changes in bacteria grown in peptone and resuspended in a solution containing only NaCl, phosphate and either butyrate or acetate were also similar to those observed in bacteria grown and resuspended in butyrate or acetate media. The same applied for bacteria, grown and resuspended in acetate medium gassed with H_2.

The dark oxidation of NAD(P)H_2 followed approximately the kinetics of a first order reaction with acetate and succinate-grown Rhodospirillum. For butyrate-grown bacteria the kinetics usually were more like that of a higher order (between 1 and 2) with respect to reduced coenzyme. However, the course of the reaction may have been influenced by an inhomogeneity of the bacterial population.

The quantum efficiency observed was of the same order of magnitude for differently grown bacteria: quantum requirements for malate and butyrate-grown Rhodospirillum are summarized in Table 3.2. Table 3.2 and Figs. 3.4 and 3.6, which show a

TABLE 3.2

Quantum requirement at different light intensities of light-induced NAD(P) reduction in Rhodospirillum rubrum.

Sample	Light intensity	1/ϕ (hν/eq.) and duration of illumination (sec).					
1, butyrate, 2 d.	9.1	7.0	(0)	5.5	(15)		
1, butyrate, 2 d.	5.3	4.3	(0)	2.9	(3)	9.2	(92)
1, butyrate, 2 d.	0.69	1.7	(0)	1.5	(4)	3.5	(197)
2, butyrate, 2 d.	4.7	4.6	(0)	2.5	(6)	4.3	(18)
2, butyrate, 2 d.	0.87	7.3	(0)	3.5	(10)	2.9	(45)
3, malate, 1 d.	9.6	4.0	(5)	5.3	(7)		
3, malate, 1 d.	2.5	5.1	(7)	3.7	(12)	4.4	(26)
3, malate, 1 d.	0.76	9.5	(13)	13	(24)		
4, malate, 2 d.	5.6	2.5	(6)	4.5	(12)	6.4	(24)

Butyrate- and malate-grown 1 or 2 days old bacteria were used in a 4% suspension contained in a 1 mm vessel. The light intensities are given in 10^{-9} einstein/(sec cm^2). The numbers in parentheses in the last three rows indicate the duration of preceding illumination. The quantum requirements 1/ϕ were calculated as in Figs. 3.4 and 3.6 from the slope of the time curves of the absorbancy at 340 mμ.

decrease of the quantum efficiency after prolonged illumination, give data obtained for relatively short illumination periods (of the order of 1 min or less). Experiments of longer duration suggested a several times lower rate of light-induced NAD(P) reduction after prolonged illumination of moderate intensity. After several min of illumination in many experiments no measurable absorption decrease was observed upon darkening. Part of these experiments were done with bacterial suspensions directly transferred from the culturing tubes to 1 cm absorption vessels; this indicates that the effect was not caused by the centrifugation or dilution with fresh medium applied in the other experiments. Sometimes small absorption shifts were observed, but it is uncertain whether these were caused by NAD(P) or by another compound.

3.3.4 The fluorescence yield of $NAD(P)H_2$ in Rhodospirillum rubrum

The total absorption increase of a 4% suspension of 2 days old malate-grown Rhodospirillum was measured upon illumination with a high intensity (9.5×10^{-9} einstein/(sec cm^2)) of light of 840 mμ. Under the same conditions the fluorescence increase was measured for the same suspension by means of the attachment of Fig. 2.7.A. The wavelength of exciting radiation was 340 mμ, obtained by means of a xenon arc. The fluorescence increase occurring by adding to the suspension a small amount of a $NADH_2$ solution of known concentration was also measured.

It was observed that the fluorescence increase upon illumination was 0.33 times that caused by the addition of $NADH_2$, and that the absorption increase caused by the light was only 0.28 times the calculated absorption increase due to added $NADH_2$. This indicates that the fluorescence yield of the coenzyme in the cells is 1.2 times that of free $NADH_2$. In this calculation it was assumed that "flattening effects" were negligible (§ 3.2). Also no corrections were made for the effect that the emission spectrum of $NAD(P)H_2$ in Rhodospirillum (75) is somewhat shifted with respect to that of free $NADH_2$, since this effect was estimated to be only a few percent with the filter combination applied. The fluorescence yield of reduced coenzyme in the cell is somewhat higher than calculated, because of the absorption of excitation and fluorescence radiation within the cell where the fluorescing $NADP(H_2)$ is located, but the effect was estimated to be not higher than about 5% (cf. § 3.2).

The cause of this rather low fluorescence yield of $NAD(P)H_2$ in Rhodospirillum is uncertain. Specific binding to enzymes (224) as well as non-specific binding (96, 21) may enhance the fluorescence yield of reduced coenzyme considerably compared to that of free $NAD(P)H_2$. It is possible that the fluorescence yield of the reduced coenzyme is decreased in intact cells of Rhodospirillum by resonance transfer of energy (see ref. 67) to carotenoids and bacteriochlorophyll, if the distance between

the $NAD(P)H_2$ molecules and the pigment molecules is not too large, since the emission spectrum of $NAD(P)H_2$ and the absorption spectra of the photosynthetic pigments partly overlap in the blue region. However, experiments to measure the amount of energy transfer by comparing the fluorescence yield of bacteriochlorophyll *in vivo* upon excitation with light of 340 mμ with the amount of photoreduced $NAD(P)H_2$ formed were not successful, because of interfering changes of bacteriochlorophyll fluorescence upon illumination.

3.3.5 NAD(P) reduction by Rhodopseudomonas spheroides

The kinetics of NAD(P) reduction in Rhodopseudomonas spheroides (Fig. 3.7) in principle followed the same pattern and the maximum efficiency observed was roughly the same as with

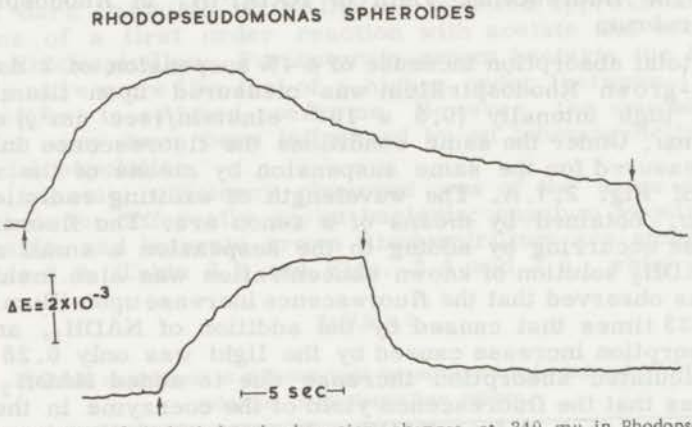

Fig. 3.7 Kinetics of light-induced absorption changes at 340 mμ in Rhodopseudomonas spheroides. The bacteria were grown and examined in the modified medium of Cohen-Bazire et al. as indicated in the text. Illumination was effected by a band around 838 mμ. The intensity was 10.9×10^{-9} einstein/(sec cm^2). The other experimental conditions were the same as in Fig. 3.3.

Rhodospirillum. We did not measure precisely whether, as in Rhodospirillum rubrum, the rate of dark reoxidation only depended on the concentration of $NAD(P)H_2$. Table 3.3 summarizes a few quantum requirements for coenzyme reduction, measured with bacteria grown in the modified medium of Cohen-Bazire *et al.* (see § 3.2). Experiments with malate-grown bacteria indicated only a relatively low rate of light-induced NAD(P) reduction after a prolonged period of illumination. Upon illumination with light of 860 mμ of an incident intensity of 3.8×10^{-9} einstein/(sec cm^2), a rate of reduction, as indicated by the rate of reoxidation upon darkening, was observed, which was only 27% after 1 min and 15% after 55 min of the maximum rate, which occurred about 7 sec after onset of illumination.

TABLE 3.3

Quantum requirement of light-induced NAD(P) reduction in Rhodopseudomonas spheroides.

Light intensity	Duration of illumination	1/φ (hν/eq.)
10.9	0	5.7
10.9	5	3.1
10.9	11	4.1
10.9	31	3.8
2.7	4	9.3
2.7	11	2.2
2.7	36	3.1

The bacteria were grown and examined in the modified medium of Cohen-Bazire and coworkers (see § 3.2); they were used, 1 day old, in a 4% suspension contained in a 1 mm absorption vessel. The light intensities are given in 10^{-9} einstein/(sec cm^2). The quantum requirements 1/φ were calculated from the rate of increase or decrease of absorbancy at 340 mμ as in Table 3.2.

3.3.6 The quantum requirement of cytochrome oxidation

As first shown by Duysens, illumination of photosynthetic bacteria causes one or more cytochromes to become oxidized, as indicated by the light-induced spectral changes in the violet and green region (68, 73, 47, 48, 206, 229, 197, 208, 199). The rapidity of the changes (cf. ref. 52) and the observation that one of the cytochromes (in Chromatium) is oxidized at the temperature of liquid nitrogen and some of the other cytochromes at temperatures well below 0°C (51, 197-199, 250) indicate that the oxidation of these cytochromes is a reaction closely associated to the primary light reaction, and that the cytochromes are oxidized directly by oxidized bacteriochlorophyll (67, 71, 10, 56, 251). The oxidized cytochromes may in part be intermediates in the oxidation of substrate, and may partly function in so-called "cyclic" photosynthetic phosphorylation by a reaction with reduced photoproducts coupled with the production of ATP (229, 22, 151, 20, 23, 197-199). In several species of algae and in chloroplasts of higher plants an oxidation of cytochromes upon illumination has also been observed; as will be discussed in Chapter IV, the role of cytochromes in green plant photosynthesis is probably partly similar to that in bacterial photosynthesis.

Since the experiments reported in the previous sections indicated that a relatively high rate of coenzyme reduction only occurred for a limited period shortly after onset of illumination, we measured the kinetics and rate of cytochrome oxidation in R. rubrum under the same conditions as applied for the measurement of NAD(P) reduction, in order to compare the time courses of these processes.

At not too high intensity in R. rubrum suspended in nutrient medium the most prominent band in the difference spectrum in the blue region was a negative one at 428 mμ, in agreement with earlier findings (68, 73). For bacteria taken from sample 3 of Table 3.2 (grown and resuspended in malate medium), the initial rate of absorption decrease at this wavelength upon illumination with light of 860 mμ corresponded to a quantum requirement for cytochrome oxidation of about 3 to 4 quanta per equivalent. A specific absorption coefficient of 70 cm^{-1} meq^{-1} l^{-1} (cf. refs. 207, 140) at 428 mμ (reduced minus oxidized) for the cytochrome was assumed. The quantum number is only an approximate one: the measurements were less accurate than those on NAD(P) reduction, because the absorption changes were smaller and more rapid than those at 340 mμ and because more than one cytochrome changes its absorption at 428 mμ. The quantum requirement was independent of light intensity in the range of 0.3 to at least 3×10^{-9} einstein/(sec cm^2). At higher intensity the rate of absorption change was too high to be measured with reasonable precision. Experiments with butyrate-grown Rhodospirillum, the same as sample 2 of Table 3.2, indicated roughly the same quantum requirement. At 1.3×10^{-9} einstein /(sec cm^2), the initial rate of absorption increase at 428 mμ upon darkening after 2 sec to 30 min of illumination was found to be about 2 times lower and that at 420 mμ about 1.2 times higher than the initial rate of absorption decrease at these wavelengths upon onset of illumination.

An interpretation of these results is difficult because of the complicated reaction kinetics (cf. refs. 73, 48) and, since at least two cytochromes are involved, would require more experimental data. However, the experiments indicate that, in contrast to NAD(P) reduction, an efficient oxidation of cytochrome occurs immediately upon illumination (see also ref. 52), and that a high turnover rate of *c* and *b*-type cytochromes is maintained during continued illumination. This suggests that the primary light reaction runs at an approximately constant rate during illumination, and, since oxidized and reduced products of this reaction have to be produced in stoichiometric amounts, indicates that during prolonged illumination an increasing part of the reduced equivalents (or electrons) are not used in the reduction of NAD(P), but take part in other reactions, possibly also in "cyclic" phosphorylation.

3.3.7 Inhibition by HOQNO and fluoroacetate

HOQNO, an inhibitor of, amongst other things (163), cyclic photophosphorylation (229, 22) did not or only partly inhibit the light-induced reduction of NAD(P) in Rhodospirillum. Fig. 3.8 shows the rather complex results of rate measurements with butyrate-grown bacteria. At 4 sec after onset of illumination the highest concentration applied (1.2×10^{-5} M) gave about 60% inhibition; after 15 sec no substantial effect was caused by any

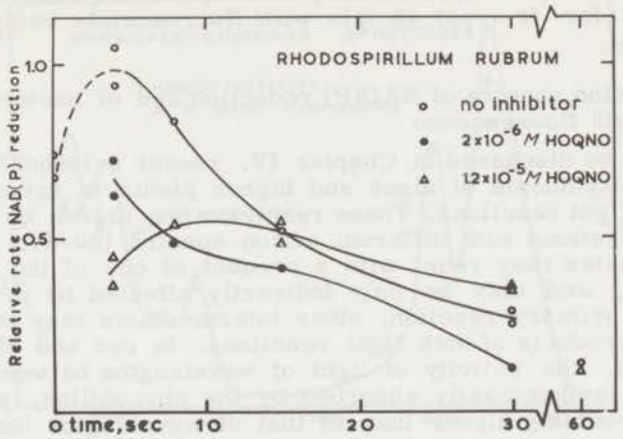

Fig. 3.8 Relative rates of light-induced NAD(P) reduction in 1 day old butyrate-grown Rhodospirillum, suspended in fresh medium in the presence of different concentrations of HOQNO. The 0.3% suspension was contained in a 1 cm cuvette; the light intensity was 4.5×10^{-9} einstein/(sec cm^2). $NAD(P)H_2$ was measured, by means of the set-up of Fig. 2.7A, as the blue fluorescence, excited by light of 340 mμ.

concentration of the inhibitor. After 30 sec HOQNO caused partial inhibition at low and a slight stimulation at high concentration. Confirming Smith' and Baltscheffsky's (229) results we found that the maximum of the absorption difference spectrum in the Soret region of the cytochromes shifted from 428 to about 422 mμ in the presence of 10^{-6} M HOQNO, which indicated that the inhibitor penetrated into the cell.

In the presence of inhibitor the rate of coenzyme reduction was less reproducible and more strongly dependent upon the preceding light-dark regime than with unpoisoned bacteria. However, also in experiments with other samples of malate and butyrate-grown bacteria at most a partial inhibition was observed even at a concentration of 10^{-5} M or higher.

Fluoroacetate, an inhibitor of the aconitase reaction of the tricarboxylic acid cycle, has been reported to inhibit, at a concentration of 8.3×10^{-4} M, the photosynthetic assimilation of acetate and butyrate by Rhodospirillum rubrum, but only slightly that of malate and succinate, while the oxidative dark assimilation of all these acids is nearly completely inhibited (86). Measurement of light-induced NAD(P) reduction revealed only a slight inhibition by fluoroacetate in the presence of malate, acetate or butyrate. E.g. a concentration of 1.6×10^{-3} M gave about 30% inhibition of the maximum rate of coenzyme reduction by malate-grown Rhodospirillum rubrum and did not inhibit this rate in acetate-grown bacteria; 5×10^{-3} M inhibited NAD(P) reduction by butyrate-grown bacteria by about 20%. The experiments were

performed with 2 days old bacteria at a light intensity of 3.0×10^{-9} einstein/(sec cm^2). The bacterial suspension was incubated for at least 40 min with fluoroacetate before measurement.

3.3.8 Action spectra of NAD(P) reduction and of bacteriochlorophyll fluorescence

As will be discussed in Chapter IV, recent evidence indicates that photosynthesis of algae and higher plants is driven by two primary light reactions. These reactions are driven by different pigment systems with different action spectra (80-84, 6). Some intermediates may react with a product of one of the two light reactions, and may be only indirectly affected by products of the other primary reaction; other intermediates may react with the photoproducts of both light reactions. In red and blue-green algae e.g. the activity of light of wavelengths between 550 and 650 mμ, predominantly absorbed by the phycobilins, was found to be appreciably higher than of that of wavelengths longer than 650 mμ, in the region of chlorophyll *a* absorption, in effecting photosynthesis and chlorophyll fluorescence. On the contrary, the action spectrum for intracellular coenzyme reduction and cytochrome oxidation showed a higher activity of chlorophyll *a* than of the phycobilins.

It was concluded that the fluorescent chlorophyll *a* belongs for the main part to a photosynthetic pigment system (system 2) which is different from that (system 1) causing NADP reduction and cytochrome oxidation (see Chapter IV). In order to determine whether the active absorption by the different types of bacteriochlorophyll (see ref. 67) and by the carotenoids is the same in effecting NAD(P) reduction as in exciting bacteriochlorophyll fluorescence, points of the action spectra for both light processes were determined for the same suspension of Rhodopseudomonas spheroides.

Bacteriochlorophyll fluorescence was measured by means of the set-up of Fig. 2.7.B. In order to minimize uncertainties due to light absorption by the pigments and to keep self-absorption of chlorophyll fluorescence small, a dilute suspension (0.7%) contained in a 1 mm vessel was used and the fluorescence was filtered by a filter transmitting above about 900 mμ. The fluorescence was a linear function of the incident intensity under the conditions applied. NAD(P) reduction was measured as an increase in absorption at 340 mμ. The same monochromator with xenon arc provided actinic light for causing NAD(P) reduction and radiation for exciting bacteriochlorophyll fluorescence; for the measurement of fluorescence the light was chopped at 50 Hz.

The results are given in Fig. 3.9. The points are plotted as the reciprocal of the number of incident quanta per sec required to bring about the accumulation of a certain amount of $NAD(P)H_2$ in a certain time or to excite a certain amount of fluorescence. It appears that the action spectra of both processes are in good

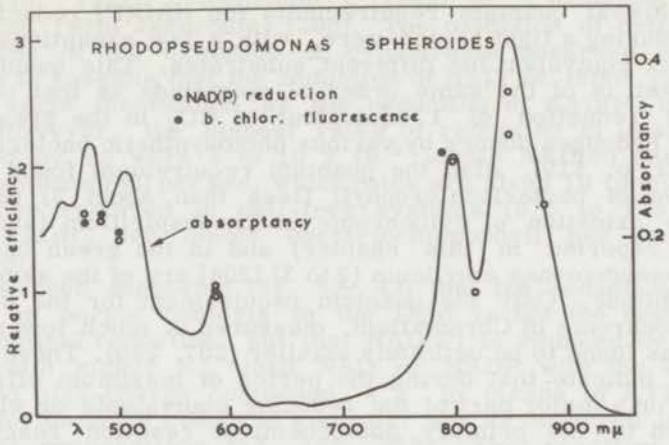

Fig. 3.9 Comparison of the efficiency of light of different wavelengths in causing NAD(P) reduction and bacteriochlorophyll fluorescence in Rhodopseudomonas spheroides. The bacteria, grown and examined in butyrate medium, were in a 0.7% suspension, contained in a 1 mm quartz vessel. The half-width of the actinic and of the exciting beams was about 7 mμ. The relative efficiencies for both light processes are given in arbitrary units; the average values at 590 mμ have been made to coincide.

approximation proportional to each other in the spectral regions where bacteriochlorophyll and the carotenoids absorb. This indicates that in purple bacteria the same photochemical system of pigments is responsible for both processes, and is consistent with the hypothesis that bacterial photosynthesis is driven by only one light reaction.

The relatively high efficiency of carotenoids in effecting bacteriochlorophyll fluorescence is in agreement with earlier measurements by Goedheer on the same species (116).

3.4 *Discussion*

The results reported in this chapter indicate that in Rhodospirillum rubrum and Rhodopseudomonas spheroides a large pool of NAD or NADP is reduced upon illumination. For R. rubrum the amount of NAD(P) which could be reduced in strong light was roughly one-tenth of that of bacteriochlorophyll. The reduction was observed in bacteria, grown and examined in organic media in the presence of various organic acids as substrates. For these bacteria a high efficiency of reduction was observed during a short period (in the order of half a minute) at the beginning of a light period. The available evidence indicates that after longer illumination a decrease of the efficiency occurred to considerably lower values. In many experiments no indications

for a light-induced coenzyme reduction were found after a few min of illumination.

The lowest quantum requirements for NAD(P) reduction observed during a light period were, with a few exceptions, about 2 to 3 per equivalent for different substrates. This quantum requirement is of the same order of magnitude as that observed for the reduction of 1 equivalent of CO_2 in the presence of various hydrogen donors by various photosynthetic bacteria (256, 177, 112, p. 119). Also the quantum requirement found for the oxidation of bacteriochlorophyll (less than about 3) (251, 57) and the oxidation of cytochrome in Rhodospirillum (about 3 to 4, as reported in this chapter) and in the green bacterium Chloropseudomonas ethylicum (2 to 3) (208) are of the same order of magnitude. Only the quantum requirement for the oxidation of a cytochrome in Chromatium, measured at much lower intensity, was found to be definitely smaller (207, 250). These observations indicate that during the period of maximum efficiency, a large or a major part of the reducing equivalents or electrons produced by the primary photochemical reaction reacts with NAD(P).

The kinetics of absorption and fluorescence changes upon darkening, as illustrated in the third curve of Fig. 3.3 suggest that coenzyme reduction goes on during a fraction of a second after the light is shut off. This indicates that the reduction of NAD(P) is not a primary photochemical process, but that it is reduced in a "dark" reaction with an unknown reduced intermediate (cf. ref. 52). The same is also indicated by the temperature dependency (79) of the reduction rate at high light intensity and, with Chromatium, by the influence of poisons and previous heating (204).

After prolonged illumination of intact cells, little or no reduction of coenzyme was observed with various substrates present, in spite of the fact that in the media used and in continuous light rapid growth and thus photosynthesis and high cytochrome turnover occurred. This indicates that in continuous light the light-induced reduction of NAD(P) is only a minor reaction quantitatively and that the major fraction of the electrons generated by the primary photochemical reaction is used in another process, e.g. the production of ATP by cyclic phosphorylation, in which reaction cytochromes probably participate (229, 22, 23, 197, 198, 151). A light-induced phosphorylation of ADP by an illuminated suspension of chromatophores of R. rubrum and Chromatium has been reported and studied by several investigators (106, 107, 113, 22, 23, 20, 246, 229, 9, 151, 200, 142). This phosphorylation was of the "cyclic" type (i.e. without the concomitant accumulation of reduced and oxidized photoproducts) and was found to proceed without added cofactors (except for Mg^{++} ions), although at high light intensity it was accelerated by PMS (23, 20) and slightly by vitamin K_3 (113). A light-induced reduction of added NAD was also observed *in vitro*. The reduction was accompanied by the oxidation of added $FMNH_2$ (107), succinate

(107, 246, 142), cytochrome *c* (245) or 2,6-dichlorophenol indophenol (with ascorbate) (200). The oxidation of cytochrome *c* reminds of the oxidation of cytochrome observed in intact cells. It is possible that the reduction of NAD *in vitro* proceeds by the same mechanism as the reduction of NAD(P) in intact cells. However, the highest rates of reduction in intact cells observed by us were 5 to 10 x 10^{-2} μmole NAD(P) per μmole bacteriochlorophyll per sec, which rates are about 10 times higher than those reported in cell-free systems (0.5 to 1.5 x 10^{-2} μmole $NADH_2$ per μmole bacteriochlorophyll per sec) (107, 246, 200).

It has been suggested (52, 53, 42) that the reduction of coenzyme in purple bacteria in the light is not the result of a photochemical reduction, but that NAD(P) is reduced by a substance with higher E'_0 than NAD or NADP with the aid of a so-called high-energy compound, e.g. by means of ATP and a succinate-fumarate couple in a similar way as observed in mitochondrial preparations (53, 185, 219). The reaction: NAD + succinate $\longrightarrow$ $NADH_2$ + fumarate, coupled with the hydrolysis of ATP, would then proceed at the expense of photochemically generated ATP. However, the observation that in intact cells NAD(P) reduction also occurs in the presence of a relatively high concentration of HOQNO, which, at even lower concentration, inhibits cyclic phosphorylation in bacterial extracts (229, 22) and probably also in intact cells (229, 198), indicates that the reduction is not an (indirect) result from ATP generation, but results from a photochemical oxidation reduction reaction. Frenkel (213) has summarized evidence based upon *in vitro* experiments against the suggestion that NAD(P) is reduced with the aid of ATP: the unability of ATP to induce NAD or NADP reduction in chromatophore preparations in the dark and the observation that NAD reduction and ATP production can proceed simultaneously in the light at different rates, dependent upon the experimental conditions. A further argument may be taken from the experiments of Nozaki *et al.* (200) who observed in the presence of HOQNO upon illumination an *in vitro* reduction of NAD with 2,6-dichlorophenol indophenol and ascorbate as electron donor, coupled with a stoichiometric production of ATP. These results, however, have been criticized by Bose and Gest (43). Recently Horio *et al.* (142) reported inhibition of NAD reduction by ATP, ADP and pyrophosphate, which inhibitions amongst other things were explained by the assumption of complexes of a reducible enzyme with NAD and ADP as intermediates in NAD reduction and cyclic phosphorylation. The observations of Nozaki *et al.* (200) that the light-induced reduction of NAD in the presence of succinate is inhibited by HOQNO suggests that the mechanism of NAD reduction is different from that *in vivo*, that in the intact cell other intermediates than succinate act as electron donor, or that the chromatophore preparation was damaged or was deficient in some catalyst or substrate.

Evidence that the reduction of coenzyme is closely connected

to the primary photochemical process is also given by the observation that the rate and kinetics of the reaction are similar for bacteria grown and examined in the presence of different organic substrates, in the presence of H_2, CO_2 and inorganic salts, and in the presence of H_2 in acetate medium. Consistent with this is also the absence of a marked inhibition by fluoroacetate.

Experiments of Stoppani *et al.* (233) with C^{14}-labelled CO_2 showed that at 8°C in Rhodopseudomonas capsulatus phosphoglyceric acid was the compound which was most heavily labelled after a short time fixation of CO_2 in the light in the presence of H_2. From this, and from the labelling pattern of other compounds they concluded that the reduction of CO_2 proceeds mainly by means of the reductive pentose phosphate cycle under these conditions. The same conclusion was obtained more recently for Chromatium grown in the presence of CO_2 as carbon source (109, 144).

Glover *et al.* (115) also reported in "resting" suspensions of R. rubrum a labelling of phosphoglyceric acid in the light in the presence of $C^{14}O_2$ and of H_2 or acetate, but, unlike Stoppani *et al.* found no labelled hexoses or hexose phosphates. The fixation pattern of C^{14} from acetate in the absence of nitrogen donors and growth factors differed from that from CO_2: 90% of C^{14} from acetate was recovered in fatty material. Elsden and Ormerod (86) concluded from inhibitor experiments with R. rubrum that in the light acetate and pyruvate were oxidized via the tricarboxylic acid cycle (cf. ref. 85), but not malate and succinate, and postulated a synthesis of cell material via phosphoenolpyruvate and triose phosphate from these acids. More recently evidence was obtained for the operation of the glyoxylic acid cycle in Rhodopseudomonas capsulatus and R. palustris (172) and Chromatium (183, 109). There are also indications for a different route for acetate oxidation in R. spheroides (242). Stanier and coworkers (231) demonstrated in starved cells of R. rubrum a direct incorporation of acetate, butyrate and succinate into cell material. Acetate and butyrate were converted mainly to the reserve substance poly-β-hydroxybutyrate and most of the succinate into polysaccharide. According to other authors (130, 110) the synthesis of poly-β-hydroxybutyrate takes only place in the absence of bicarbonate. The fixation of CO_2 was quantitatively less important (cf. refs. 115, 210). The findings of Stanier and coworkers suggested to the authors that under these conditions the action of light would, for the main part, be restricted to the generation of ATP. It was e.g. suggested that with acetate of each 9 molecules assimilated, one was oxidized by means of the tricarboxylic acid cycle and the remaining ones were converted to polymer. By assuming that the $NADH_2$ produced in the tricarboxylic acid cycle was used for polymer synthesis, it was calculated that only one NAD molecule had to be reduced photochemically for 9 acetate molecules assimilated and that the major light reaction would be cyclic

phosphorylation. A partly similar mechanism for acetate conversion was indicated by experiments with the green bacterium Chlorobium limicola (218). In Rhodospirillum rubrum photophosphorylation was suggested to be the only light reaction in the presence of H_2 and acetate (231). As mentioned, however, in our experiments there was little difference between the rates of photoreduction of coenzyme in acetate medium in the presence and in the absence of H_2. Losada *et al.* (183), mainly on basis of experiments with cell-free extracts of Chromatium, similarly concluded that in the presence of H_2 and CO_2 the action of light in this bacterium is restricted to the production of ATP.

The above-mentioned experiments are only part of those reported in the literature (cf. ref. 211), but provide sufficient evidence, that, at least under certain conditions and in the presence of organic substrate, bacterial photosynthesis, contrarily to van Niel's hypothesis (195, see also ref. 196), does not proceed solely as a light-induced reduction of CO_2 and a concomitant oxidation of substrate, but that a quantitatively important part of the light-induced cellular syntheses may occur by way of a direct incorporation of substrate. The type of metabolic reactions prevailing probably depends strongly upon the state of the cell and the experimental conditions. Since we were primarily interested in the rate of light-induced coenzyme reduction under conditions of rapid photosynthesis and growth, most experiments were done with cells taken from rapidly growing cultures and resuspended in medium of the same composition as that in which the cells had been grown. In some experiments the cells were taken from the culture tube without further treatment.

The hypothesis of Stanier *et al.* and Losada *et al.* that the main function of the light is the generation of ATP, is consistent with our results that during steady state conditions the rate of light-induced NAD(P) reduction is probably low. However, the cause of the high initial rate of reduction and the mechanism by which this rate decreases during illumination are obscure. One might assume that the process of bacterial photosynthesis in the presence of organic substrate needs only little photochemically reduced $NAD(P)H_2$. Then one might expect $NAD(P)H_2$ to accumulate in the light, so NAD(P) reduction would be stopped by lack of NAD(P) and the utilization of light energy would be directed to cyclic ATP production. This simple explanation, however, does not hold, because the amount of NAD(P) in the cells decreases during continued illumination. It might be speculated that illumination causes in the cell an exhaustion of ATP and an accumulation of ADP, which stimulates cyclic phosphorylation, and decreases the rate of coenzyme reduction. That cyclic phosphorylation and NAD reduction may be competitive reactions was demonstrated by Frenkel (107) in a cell-free extract. It is also possible that the rate of cyclic phosphorylation is increased and that of coenzyme reduction decreased because the first reaction is stimulated by an accumul-

ation of oxidized compounds produced in the light. This accumulation could be caused by a relatively too slow dark reaction of e.g. an oxidized cytochrome. Some support for this hypothesis is given by the gradual accumulation of oxidized cytochromes which was observed upon continued illumination of intact Rhodospirillum rubrum (73, 48) and which we found also under the conditions of our experiments.

Another possibility would be that there is a second, much smaller pool of NAD or NADP in the bacteria, which, unlike the large pool, has a high turnover rate also after a long period of illumination. This pool might cause absorption changes which are too small to be identified and measured by the present method. At present our results do not yield convincing evidence against or in favor of this hypothesis.

Since the fluorescence and absorption spectra of $NADPH_2$ and $NADH_2$ are the same, it cannot be concluded from our experiments whether NADP or NAD is reduced in the intact cell. A NAD-specific glyceraldehyde-3-phosphate dehydrogenase has been found in photosynthetic bacteria (227), and in cell-free systems upon illumination only NAD is reduced (107, 245, 246, 200), unless PPNR (222) from spinach is added to the reaction system (244), but from R. rubrum and Chromatium a photoreductase was isolated, which preferentially catalyzes the light-induced reduction of NADP in spinach chloroplasts (184).

As shown in Fig. 3.9, in Rhodopseudomonas spheroides the activities of light at various wavelengths in effecting coenzyme reduction and in exciting bacteriochlorophyll fluorescence are approximately proportional to each other. This indicates that one photochemical pigment system is responsible for both light processes and is in agreement with the hypothesis that in purple and green bacteria, which in contrast to algae and higher plants are unable to evolve oxygen, only one pigment system is active in photosynthesis. It must, however, be emphasized that the available evidence for this hypothesis (shortly summarized in Chapter IV), does not definitely prove that only one photochemical system is present, since quantitatively and qualitatively similar results at different wavelengths can also be explained by the assumption that two pigment systems are present with the same action spectra.

CHAPTER IV

BLUE-GREEN ALGAE

4.1 *Introduction*

As mentioned in Chapter I, like in purple bacteria, there is spectrophotometric evidence for a reduction of NAD(P) in intact algae upon photosynthetically active illumination. Further it has been found that isolated chloroplasts from various species were able upon illumination to reduce added NADP or NAD with concomitant evolution of oxygen. The experiments to be reported in this chapter were performed in order to obtain information about the role of photoreduction of coenzyme in green plant photosynthesis by quantitative measurements *in vivo*.

Until recently it was usually assumed that photosynthesis of green plants was brought about by one "primary oxidation reduction reaction", activated by chlorophyll *a*. Chlorophyll *a* received its energy partly by the absorption of light quanta, partly by inductive resonance from accessory pigments (66, 67). To explain the relatively low activity of light absorbed by chlorophyll *a* in effecting photosynthesis (66, 67, 123) and chlorophyll *a* fluorescence (66, 67) in most red and blue-green algae, the existence of a second type of chlorophyll *a in vivo* had to be postulated (66, 67), which was inactive or less active in these processes and received little excitation by energy transfer from the phycobilins. In the green alga Chlorella it had been found that light of wavelengths longer than about 680 mμ absorbed by chlorophyll *a* was less active in photosynthesis than light of shorter wavelengths (89).

More recent experiments of Emerson and others (91, 92, 39) indicated that light of different wavelengths acted not only quantitatively but also qualitatively differently in photosynthesis. It was found that e.g. in Chlorella pyrenoidosa the rate of photosynthesis was larger upon simultaneous illumination with two beams of about 700 and 650 mμ respectively than the sum of the rates when the two beams were given separately ("Emerson effect"). Although these and related experiments clearly demonstrated that the rate of photosynthesis is affected by more than one light process, they gave little evidence about the mechanism of these processes and specifically did not demonstrate that two major primary reactions occurred.

Evidence for the existence and function of two major photochemical systems was obtained in experiments of Kok *et al.* (168, 169) and of Duysens and coworkers (80-84, 6, 8). Experiments of the latter authors on, amongst other things, light-induced cytochrome oxidation and reduction and photosynthesis in intact cells of Porphyridium cruentum (80-84) and experiments,

to be reported in this chapter, on photosynthesis, light-induced coenzyme reduction and cytochrome oxidation in Anacystis nidulans (6), were explained by the following scheme:

$$H_2O \longrightarrow (\text{system 2}) \longrightarrow Q \longrightarrow PQ \longrightarrow \text{cytochrome} \longrightarrow P \longrightarrow (\text{system 1}) \longrightarrow NAD(P)H_2 \longrightarrow CO_2$$

The evidence pertaining to this scheme will only be briefly and partly summarized here; a more extensive discussion of some of its aspects and of evidence obtained by others will be more conveniently given later in this chapter. Two light reactions, driven by system 1 and system 2, were postulated. System 1 reduces NAD(P) and oxidizes a substance P; system 2 reduces Q and produces O_2 by oxidation of water. Oxidized P reacts with reduced Q in dark reactions via a cytochrome and PQ (plastoquinone or a related compound). The various compounds of the chain are more or less arbitrarily represented by the oxidized or reduced form of an oxidation reduction couple. The arrows indicate the direction of hydrogen or electron transport. The scheme is not complete, e.g. phosphorylations have been omitted (cf. refs. 184, 261). P, the pigment "P 700", was included (81) on basis of studies of Kok and coworkers (168, 169). The function of Q was postulated on basis of experiments on chlorophyll fluorescence (83, 84). Plastoquinone (PQ) has rather recently been isolated in relatively large amounts from algae and chloroplasts of higher plants (32, 180) together with smaller amounts of related compounds (126). On basis of evidence obtained with isolated chloroplasts (e.g. a stimulation of the Hill reaction and other photochemical activities (32, 241, 126)) it has been postulated to be a component of the photosynthetic chain (193, 241, 267, 258). By means of difference spectroscopy in the ultra violet region of intact Anacystis cells, we recently obtained direct evidence for an efficient reduction by light absorbed by system 2 and oxidation by light absorbed by system 1 of plastoquinone or of a quinone with similar absorption spectrum (8). System 1 contains weakly fluorescent, system 2 contains more strongly fluorescent chlorophyll *a* (cf. refs. 66, 67). DCMU, N-ethylurethane and hydroxylamine prevent the reduction of cytochrome by system 2, but not the oxidation by system 1; DCMU inhibits the reduction, but not the oxidation of the quinone.

Basically similar hypotheses for two light reactions were also presented by others (128, 184, 266), partly on more or less speculative grounds. The evidence for the hypothesis of a parallel action of two photochemical systems (see refs. 104, 105, 3) seems to be less convincing. A discussion of the evidence is given in ref. 82.

Most experiments to be reported in this chapter were done with Anacystis nidulans, because the light-induced changes in $NAD(P)H_2$ fluorescence and absorption could be measured with better precision in this species than in other algae. Further-

more, the alga is strictly photoautotrophic (175), and, as will be shown, the two pigment systems can be distinguished rather clearly in this species. The fact that under our culture conditions the Anacystis cells were separated from each other facilitated handling of the samples and quantitative evaluation of the results.

A comparison of the rate, kinetics and action spectrum of coenzyme reduction, cytochrome oxidation and photosynthesis indicated that the photosynthetic reduction of CO_2 proceeds mainly or solely via NAD(P). The results could be explained by means of the scheme mentioned and yielded evidence about the function and pigment composition of the two pigment systems in Anacystis and other blue-green algae.

4.2 *Materials and Methods*

Anacystis nidulans (P. Richt.) Drouet and Daily was grown in the liquid medium C of Kratz and Myers (175) to which was added an amount of 0.84 g Na_2CO_3 per liter. Before inoculation the medium was gassed with air enriched with 4% CO_2.

Schizothrix calcicola (Ag.) Gom., strain TX 27, was grown in an anorganic liquid culture medium (B. Kok, personal communication). The medium was prepared by dissolving in a final volume of 1000 ml: $CaCl_2$, 60 mg; KNO_3, 500 mg; KH_2PO_4, 250 mg; $Fe_2(SO_4)_3$, 0.4 mg; Tris (Sigma buffer 7-9), 500 mg and 10 ml of a micronutrient solution. The micronutrient solution was obtained by dissolving in 1000 ml: $(NH_4)_6Mo_7O_{24}.H_2O$, 530 mg; $FeCl_3.6H_2O$, 300 mg; $ZnSO_4.7H_2O$, 66 mg; $MnCl_2.4H_2O$, 430 mg; $Co(NO_3)_2.6H_2O$, 1.5 mg; $CuSO_4.5H_2O$, 0.47 mg; H_3BO_3, 3.4 g and ethylenediamine-tetra-acetic acid disodium salt, 3 g. After autoclaving the pH of the medium was 7.7. A slight turbidity was formed, which vanished upon bubbling with air and 4% CO_2. Grown under these conditions, the alga consisted of short filaments of about 5 to 20 cells and contained roughly equal amounts of phycoerythrin and phycocyanin. It was isolated by J. Myers (Department of Zoology, University of Texas). Dr. F. Drouet (Academy of Natural Sciences, Philadelphia) kindly identified a sample of this alga, formerly only known as "TX 27".

Anabaena cylindrica Lemm. was grown in the culture medium described in ref. 1.

All algae were grown in the light of fluorescent tubes (Philips "de luxe", type 34) at an intensity of about 2200 lux, and at a temperature of 25°C. The cultures were bubbled with air enriched with 4% CO_2. Before measurement Schizothrix and Anacystis were harvested by centrifugation, transferred to fresh growth medium (see § 2.1) and gassed with a mixture of air and 5% CO_2 during an hour or longer before measurement. Dr. W. Terpstra, Utrecht, Dr. B. Kok, Baltimore and Prof. G.E. Fogg, London,

kindly provided cultures of Anacystis, Schizothrix and Anabaena, respectively.

Photosynthesis was measured polarographically as O_2 production (§ 2.6). For Anacystis the recessed electrode was used; oxygen production of Schizothrix was measured by pressing a thin layer of a paste of cells against a non-recessed electrode. Light-induced absorption changes were measured by means of the apparatus described in § 2.4, equipped with the attachment for illumination with two actinic beams of different wavelengths Fluorescence measurements were done with the set-up of Fig. 2.7.A. The absorptancy of the algal suspensions was measured and corrected for scattering as described in § 2.3. The experiments were carried out at room temperature (about 22°C).

The quantum requirements for NAD(P) reduction and cytochrome oxidation reported in this chapter were calculated from rates of the absorbancy changes at 340 and at around 420 mμ upon illumination in the algal suspensions. In order to convert these absorbancy changes to reaction rates of absorbing pigments, corrections had to be made for optical effects occurring in suspensions, as discussed in § 2.2.

Deviations from Beer's law were corrected by measuring the absorbancy of suspensions of different concentrations with opal glass. In this way it was found that for a 1 mm layer of a 1.5% suspension of Anacystis (the concentration usually applied in the experiments) the measured absorbancy had to be divided by 1.18 at 340 mμ and 1.05 at 420 mμ.

An approximate correction for the effect of "particle flattening" (§ 2.2.2) was made as follows: Assuming the cells to be spherical in shape (instead of short rods), $T'_{p\ av}(\gamma)$ was calculated by means of eq. (2.7) by counting the number of cells in a dilute (0.5%) suspension and measuring the absorbancy in a 1 mm vessel with opal glass. $T'_{p\ av}(\gamma)$ was found to be 0.95 at 340 and 0.86 at 420 mμ. As curve (4), Fig. 2.1 shows, this corresponds to a flattening factor, $dE'(\gamma)/dE_{sol}$, of 0.94 and 0.84 respectively.

The effect of "scattering flattening" was calculated for Anacystis in a similar way (§ 2.2.1). We have measured the absorbancy at 340 and 420 mμ of a 0.5% suspension in the absorption difference spectrophotometer, and also with opal glass in the Zeiss spectrophotometer. Neglecting the flattening of the latter spectrum (§ 2.3) the flattening factor $1-E''(\gamma)/p \log e$ of eq. (2.2) was calculated by means of eq. (2.1); p was calculated, assuming the cells to be spherical, by counting the number of cells in the suspension. However, this calculation only applies when the suspension is sufficiently dilute to show a linear relation between the absorbancy and the concentration (5). For a dense suspension the effect might be expected to be smaller than calculated, because repeated scattering between the particles will probably tend to make the scattered light more homogeneous, because part of the light scattered outside the angle γ by a particle will be scattered back by a second particle. Since for a 1 mm layer of a 1.5% suspension a deviation from linearity was found for the absorbancy

measured with opal glass which amounted to about 5% at 420 mμ and about 20% at 340 mμ, we have assumed the flattening to be only 90% at 420 mμ and 50% at 340 mμ of the calculated effect. In this way an estimated flattening factor of 0.89 at 420 and 0.94 at 340 mμ was obtained. If for a 1.5% suspension the effect would be the same as for a dilute one, the factors would be both 0.88, a relatively small deviation.

Taking all three effects together, we have divided the measured absorbancy changes for a 1 mm layer of a 1.5% suspension of Anacystis by 1.19 x 0.94 x 0.94 = 1.05 at 340 mμ and by 1.05 x 0.84 x 0.89 = 0.79 at 420 mμ.

4.3 *Results and Interpretation*

4.3.1 Action spectrum for photosynthesis of Anacystis nidulans

We have measured the action spectrum of photosynthesis of Anacystis, taken from cultures of different age. Fig. 4.1 shows

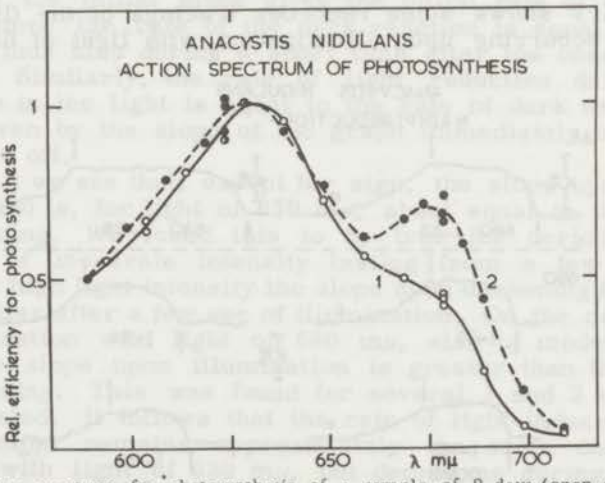

Fig. 4.1 Action spectrum for photosynthesis of a sample of 2 days (open circles) and 3 days old Anacystis nidulans (solid circles). The algae were settled in a layer of about 4 μ thickness upon the electrode of the polarograph. The half-width of the actinic beam was about 5 mμ.

the action spectra of two cultures; curve (1) has been obtained with a sample taken 2 days after inoculation from a rapidly growing culture of medium density, curve (2) with a sample from a 3 days old culture which had about reached its maximal density. In contrast to the action spectra, the absorption spectra of both cultures were nearly the same; the absorptancy at the maximum of chlorophyll *a* absorption at 675 mμ was between 90 and 95% of that at the phycocyanin maximum at 625 mμ.

The action spectra of photosynthesis show a marked difference in the activity of chlorophyll *a* at about 680 mμ; that of the younger culture shows the lowest activity of chlorophyll and resembles most that given by Kok and Hoch (168). Both action spectra, although different, show a relatively low photosynthetic activity of chlorophyll *a*, compared to that of phycocyanin. A relatively low activity of chlorophyll *a* was reported for most, but not all, species of red and blue-green algae studied so far (123, 67, 88, 124, 45). Our results show that the relative activities depend strongly upon the growth conditions.

4.3.2 Kinetics and action spectrum of NAD(P) reduction

The kinetics of the reduction of NAD(P) in Anacystis nidulans upon illumination were studied by means of fluorescence measurements in the blue region around 450 mμ. Evidence that the increase in blue fluorescence is due to reduction of coenzyme was given by Duysens and Sweep (75) and Olson and Amesz (205) by means of the emission and action spectrum of the fluorescence, in a similar way as with purple bacteria (see § 3.1).

Fig. 4.2 shows some recorder tracings of the fluorescence changes occurring upon illumination with light of 680 and 630

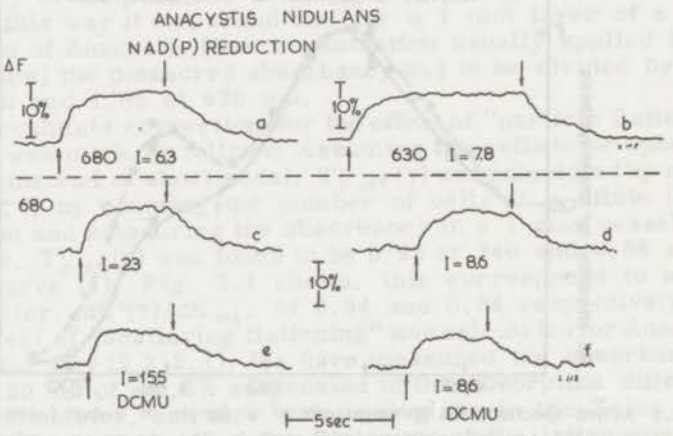

Fig. 4.2 Kinetics of the blue fluorescence, excited by irradiation of 334 mμ wavelength, of a suspension of Anacystis nidulans upon illumination with actinic light of 680 and of 630 mμ. The suspension was contained in a 1 mm quartz cuvette. An upward deflection indicates an increase of fluorescence, caused by reduction of NAD(P). Upward and downward pointing arrows indicate the beginning and the end of an illumination period. The incident intensities I of actinic light are expressed in 10^{-10} einstein/(sec cm^2). Recordings (a) and (b) were obtained with samples of a 1.2% suspension of 1 day old, the other recordings with samples of a 1.0% suspension of a 2 days old culture. The concentration of DCMU in the last 2 experiments was 1.1×10^{-5} M. The vertical line segments indicate a fluorescence difference equal to 10% of the fluorescence without actinic light.

mμ, wavelengths mainly absorbed by chlorophyll *a* and phycocyanin. The graphs indicate that the rate of reduction and oxidation of NAD(P) changes immediately upon switching on and off the light, which indicates that the light reaction also starts and stops immediately. Although filters and cuvette were selected for low fluorescence, probably part of the dark fluorescence recorded originated from the filters, cuvette and medium as indicated by experiments with cell suspensions of lower concentrations, but these effects did not contribute to the fluorescence *changes*.

In accordance with the scheme mentioned (§ 4.1) and also, as we will show, in accordance with our experiments, we assume that only one light reaction of NAD(P) occurs: its reduction. Consequently the reoxidation of $NAD(P)H_2$ is a dark reaction. As the rate of a dark reaction depends upon the concentration of the reactants, this rate may be expected not to change abruptly. The nett rate of coenzyme reduction is measured by the slopes of the fluorescence curves of Fig. 4.2. This nett rate then is the sum of the rates of the light reduction and dark oxidation. During the dark steady-state both rates are zero. At onset of illumination the initial slope gives the initial rate of the light reaction, since the rate of the dark reaction is equal to zero before and thus also during a short time after the onset of illumination. Similarly, the rate of light reduction during the steady-state in the light is equal to the rate of dark oxidation, which is given by the slope of the graph immediately after the light is shut off.

In Fig. 4.2 we see that, except for sign, the slope upon onset of illumination is, for light of 630 mμ, about equal to the slope upon darkening. We found this to be true for periods of illumination of moderate intensity lasting from a few sec up to 5 min. At high light intensity the slope upon darkening becomes a little smaller after a few sec of illumination. On the contrary, upon illumination with light of 680 mμ, also of moderate intensity, the slope upon illumination is greater than the slope after darkening. This was found for several 1 and 2 days old cultures tested. It follows that the rate of light-induced coenzyme reduction remains approximately the same during illumination with light of 630 mμ, but decreases during illumination with light of 680 mμ. These findings are consistent with the proposed mechanism of photosynthesis as will be discussed below.

Table 4.1 summarizes some of the experiments with suspensions of Anacystis taken from different cultures. It appears that quanta absorbed at 680 mμ are about 50% more active in effecting the initial NAD(P) reduction than quanta absorbed at 620 or 630 mμ; on the contrary the quanta of 680 mμ are only about half as effective in maintaining the steady-state reduction of coenzyme during an illumination period. At low intensity the rate of NAD(P) reduction was found to be a linear function of intensity for illumination with light of 630 and 680 mμ. Experiments indicated a saturation of coenzyme reduction at an incident

TABLE 4.1

Relative rates of NAD(P) reduction upon onset of illumination and of oxidation upon darkening in Anacystis nidulans

Culture	Actinic light		Reduction rate	Oxidation rate		Ratio
	λ, mμ	intensity	(R)	(O)	time	O/R.
1	630	7.8	7.4	6.4	6 sec	0.87
2	620	8.9	8.5	8.3	5 min	0.98
1	680	6.3	9.5	3.6	6 sec	0.38
2	680	6.6	10.5	3.3	5 min	0.31

The measurements were done with two different samples of Anacystis, (1) a 1.2% suspension of 1 day old algae, and (2) a 1.0% suspension of 2 days old Anacystis. The intensities of actinic light are given in 10^{-10} einstein/(sec cm^2). In the fourth column the initial rate of NAD(P) reduction upon onset of illumination is given as the percentage increase of fluorescence per sec; similarly the initial oxidation of NAD(P)H_2 upon darkening at the time indicated is given in column 5. As discussed in the text, the rate of oxidation of NAD(P)H_2 immediately after switching off the light may be assumed to be equal to the steady-state rate of light-induced NAD(P) reduction during illumination. The data represent the average of 2 to 3 measurements.

intensity of about 3 to 4 x 10^{-9} einstein/(sec cm^2) for light of a band around 630 mμ, i.e. of the same order as the intensity needed for saturation of photosynthesis, measured polarographically, but these results were very inaccurate because of the rapidity of the fluorescence changes at high intensity.

Fig. 4.3 gives the action spectrum for the initial rate of NAD(P) reduction in the region 620-720 mμ, measured with a 4 days old suspension. The spectrum is plotted as the reciprocal of the relative number of incident quanta, needed to bring about a certain initial rate of reduction at different wavelengths. It can be seen that for this suspension quanta absorbed by phycocyanin are about equally effective in NAD(P) reduction as quanta absorbed by chlorophyll *a*.

DCMU at a relatively high concentration (up to 4.3 x 10^{-5} M) had only little effect upon the initial rate of coenzyme reduction (cf. Fig. 4.2). In an experiment with a 2 days old culture in the presence of 10^{-5} M DCMU a slight inhibitory effect at high light intensities and a slight stimulating effect at low intensities was observed. E.g. upon illumination with light of 680 mμ, the initial rate of NAD(P) reduction was lowered by 20-25% at an intensity of 18.2 x 10^{-10} einstein/(sec cm^2) and stimulated by 10% at 6.[illegible] and 3.5 x 10^{-10} einstein/(sec cm^2). About the same effects occurred at 620 mμ. The rate of NAD(P)H_2 reoxidation upon darkening, measured 4 sec after onset of illumination, was much more strongly inhibited. Here DCMU at the same con-

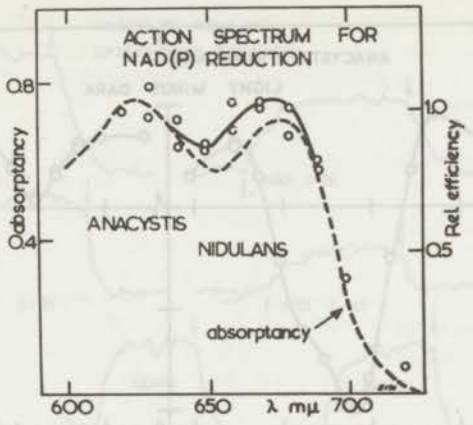

Fig. 4.3 Action spectrum for the initial rate of NAD(P) reduction of a 1.0% suspension of 4 days old Anacystis nidulans, contained in a 1 mm cuvette. Open circles represent points of the action spectrum of NAD(P) reduction; the dashed line gives the absorptancy of the suspension, corrected for scattering. The half-width of the actinic beam was 5-8 mμ.

centration gave up to 75% inhibition for high intensity illumination of 620 or 680 mμ. At a lower intensity (6.7×10^{-10} einstein/(sec cm^2) 50% inhibition was observed upon illumination with light of 680 mμ. At light intensities above about 4×10^{-10} einstein/(sec cm^2)) the rate after 4 sec was low and practically independent upon the intensity of illumination; the decrease in fluorescence for a given culture was 2.5 ± 0.3% per sec upon darkening.

The above experiments, which will be discussed later in this chapter, are consistent with the hypothesis that in Anacystis two pigment systems exist, similar to systems 1 and 2 in Porphyridium cruentum. System 1, which is responsible for the oxidation of cytochrome in Porphyridium (81, 82) and presumably also in other algae and higher plants, probably also effects the reduction of coenzyme. In order to obtain more evidence for this assumption, the pigment systems of Anacystis were further characterized by experiments on light-induced cytochrome oxidation in this species.

4.3.3 Action spectrum and kinetics of cytochrome oxidation

Upon illumination of intact Anacystis with red light, negative changes in absorption were observed in a region around 425 mμ and positive changes around 400 and above about 445 mμ. Fig. 4.4 shows the spectrum of the absorption difference between the steady-state levels with and without actinic light in the blue and violet and in the green region.

The shape of the spectrum in the region 390-430 mμ is very similar to the difference spectrum between the oxidized and the

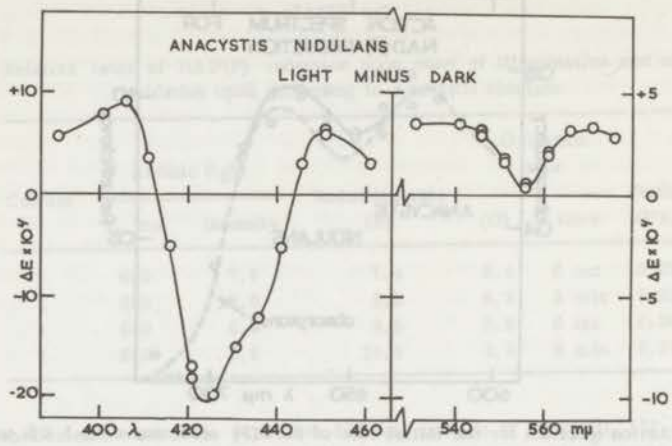

Fig. 4.4 Difference spectrum (light minus dark) of absorbancy changes upon illumination in a 2 days old, 2% suspension of Anacystis nidulans, contained in a 1 mm cuvette. The wavelength of illumination was 688 mμ, the intensity 6.5×10^{-10} einstein/(sec cm^2). Note the different scales for the two parts of the figure.

reduced state of a cytochrome, and strongly resembles that of e.g. Porphyridium cruentum (69, 82). The shoulder at 430-440 mμ and rather large positive changes at about 450 mμ (which are absent in the spectrum of Porphyridium) are probably caused by a different compound. The shape of the difference spectrum in the green region suggests that it results from two absorption changes: a negative one between 545-565 mμ and a positive one which is about the same at all wavelengths between 530 and 575 mμ. The band at 545-565 mμ suggests the disappearance of the α-band of a reduced cytochrome, as is consistent with the absorption changes in the violet region. The positive change may be caused by an increase of light scattering rather than by an absorption increase. So the absorption difference spectrum indicates that upon illumination an *f*- or *c*-type cytochrome is oxidized in Anacystis. The α-band of the reduced form has a maximum at 554 ± 1 mμ, the γ-band at 420-425 mμ. The cytochrome may be chemically similar, but is, at least in the intact cell, not identical optically to one of the cytochromes extracted from Anacystis (136) or to cytochrome *f* from parsley (62). The optical properties are similar to those of the cytochrome reacting in Porphyridium (69, 82).

Fig. 4.5 shows recordings of absorption changes at 418 mμ, where absorption changes due to other substances than the cytochrome are probably small. It can be seen that illumination with light of 680 mμ causes a much larger decrease in absorption than illumination with light of 620 mμ of about the same intensity. Addition of DCMU or N-ethylurethane to the algal suspension had little effect upon the action of light of 680 mμ, but

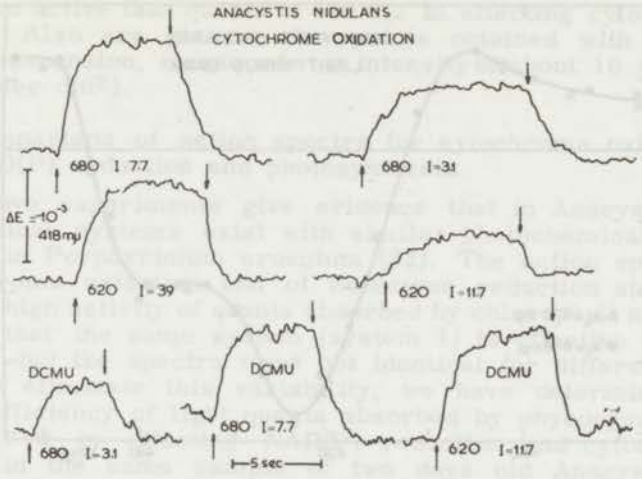

Fig. 4.5 Recording tracings of absorption changes at 418 mμ at different wavelengths and intensities of actinic light of a 2 days old, 1.5% suspension of Anacystis nidulans, contained in a 1 mm cuvette. An upward deflection (decrease of absorbancy) upon illumination indicates an oxidation of cytochrome. The bottom traces were recorded in the presence of 4.3×10^{-5} M DCMU. See also Fig. 4.2 for further details.

resulted in a striking increase of the apparent activity of light of 620 mμ. As illustrated by the three bottom recordings, in the presence of inhibitor quanta of 620 mμ were nearly as effective as quanta of 680 mμ in causing cytochrome oxidation.

Fig. 4.6 shows that the difference spectrum in the presence of N-ethylurethane is the same upon illumination with light of 678 and 625 mμ. Similar difference spectra (not shown in the figure) were obtained in the presence of DCMU.

The experiments indicate that in non-inhibited cells the oxidation of cytochrome upon illumination with light of 620 mμ is largely compensated by a simultaneous photoreduction; upon addition of DCMU or urethane the photoreduction of cytochrome apparently is inhibited, while the photooxidation is not. The same effects have been observed in Porphyridium upon illumination with light of 560 mμ (81, 82).

The spectra of Figs. 4.4 and 4.6 were obtained with samples taken from different cultures. The spectra are very similar in the region 390-425 mμ, but differ at higher wavelengths. This gives further evidence that the shoulder at 430-440 mμ is caused by a compound, different from that absorbing at shorter wavelengths; it may be similar to the substances which cause absorption changes around 430-440 mμ in Nostoc and Nitzschia (165), and in spinach chloroplasts (265).

The action spectrum of cytochrome oxidation of urethane-inhibited cells determined from the steady-state change in absorption at 420 mμ is shown in Fig. 4.7. The spectrum shows

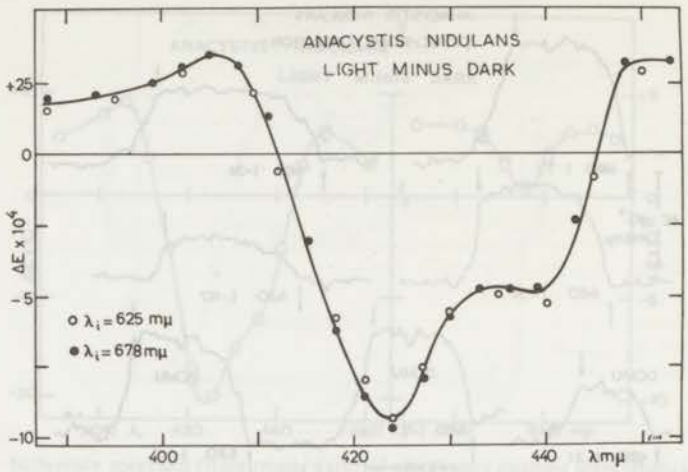

Fig. 4.6 Difference spectrum (light minus dark) of absorption changes in the blue region in 3 days old Anacystis nidulans upon illumination. The algae were in a 0.1% suspension, contained in a 1 cm cuvette, in the presence of 4.3×10^{-2} M N-ethylurethane. Points represented by open circles were obtained upon illumination with light of 625 mμ of an incident intensity of 21×10^{-10} einstein/(sec cm^2); solid circles represent measurements with light of 678 mμ of 22×10^{-10} einstein/(sec cm^2).

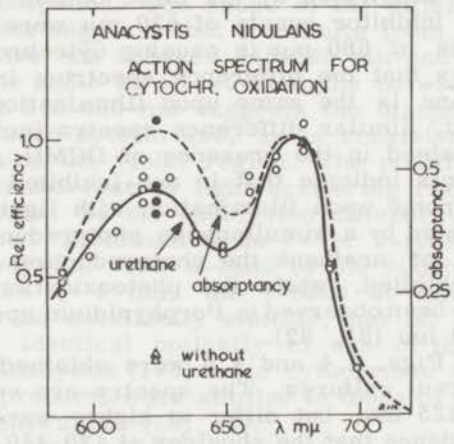

Fig. 4.7 Action spectrum for cytochrome oxidation of a 2 days old, 0.07 (volume) % suspension of Anacystis nidulans, contained in a 1 cm cuvette, measured in the presence of 4.3×10^{-2} M N-ethylurethane (open circles) and of 8.6×10^{-2} M N-ethylurethane (solid circles). Points indicated by triangles were measured in the absence of urethane at an intensity of about 10^{-9} einstein/(sec cm^2). The dashed line gives the absorptancy of the suspension, corrected for scattering. The spectra are adjusted to the same height at 680 mμ.

that for this suspension light quanta of 680 mμ are about 1.2 times more active than quanta of 620 mμ in effecting cytochrome oxidation. Also are given a few points obtained with a non-poisoned suspension, measured at an intensity of about 10×10^{-10} einstein/(sec cm^2).

4.3.4 Comparison of action spectra for cytochrome oxidation, NAD(P) reduction and photosynthesis

The above experiments give evidence that in Anacystis two photochemical systems exist with similar photochemical properties as in Porphyridium cruentum (82). The action spectrum of cytochrome oxidation and of coenzyme reduction showed a relatively high activity of quanta absorbed by chlorophyll *a*, which indicated that the same system (system 1) is effective in both reactions, but the spectra were not identical for different cultures. To eliminate this variability, we have determined the relative efficiency of light quanta absorbed by phycocyanin and chlorophyll *a* in effecting NAD(P) reduction and cytochrome oxidation in the same sample of two days old Anacystis, to which DCMU was added. The results are given in Table 4.2,

TABLE 4.2

Relative activities of light of 680 mμ, compared to that of 620 or 625 mμ in Anacystis nidulans

Culture	Type of experiment	Inhibitor	Background light, λ (mμ)	Relative efficiency 680 mμ	Pigment system
1	NAD(P) reduct.	1.1×10^{-5} M DCMU		1.47 ± 0.10	1
1	Cyt. oxidation	1.1×10^{-5} M DCMU		1.35 ± 0.06	1
1	Absorptancy			0.88	-
2	Cyt. oxidation	8.6×10^{-6} M DCMU		1.29	1
2	Photosynthesis		620	0.89	1
2	Photosynthesis		680	0.18	2
2	Photosynthesis	7.5×10^{-8} M DCMU	620	0.15	2
2	Photosynthesis	7.5×10^{-8} M DCMU	680	0.14	2
2	Photosynthesis			0.41	-

The experiments were performed with two different cultures of 2 days old Anacystis. The relative efficiency of incident quanta of 680 mμ is given, compared to that of quanta of 620 mμ (for the first three experiments) or of 625 mμ (for the other experiments). In the photosynthesis experiments without DCMU the intensities of the background radiation were 3×10^{-9} einstein/(sec cm^2) at 680 mμ and 1×10^{-9} einstein/(sec cm^2) at 620 mμ; the intensities applied for the actual measurements were between 1.7 and 3.5×10^{-10} einstein/(sec cm^2). For the photosynthesis experiments with DCMU all intensities used were about twice as high; photosynthesis at 625 mμ was about 75% inhibited by the concentration of DCMU applied. The other experimental conditions were as described already (Figs. 4.1 and 4.3).

rows 1 and 2: the relative efficiencies were found the same within the experimental error.

By means of photosynthesis measurements it is possible to determine the action spectrum of system 1 in an independent way, and also the action spectrum of system 2 (82). If it is assumed that the products made by each of the two systems are sufficiently long-lived, and that these products have to react in a fixed proportion in order to give photosynthesis, then it follows that the efficiency of light of various wavelengths is determined by the system which runs at the lowest rate (under the conditions of the experiment). If we poison system 2 sufficiently with DCMU so that this system is running at the lowest rate at all wavelengths, then the action spectrum of photosynthesis of the poisoned algae will be proportional to that of system 2. Instead of DCMU we may use a constant background of strong but non-saturating light of 680 mμ, which will presumably cause system 1 to run at a much higher rate than system 2. The efficiency of added weak light of any wavelength will then be given by the activity of system 2 at this wavelength, so that also the action spectrum of photosynthesis thus measured will be proportional to that of system 2. Analogously, the action spectrum of photosynthesis measured against a strong background of 620 mμ will be proportional to system 1.

Fig. 4.8 shows recordings of photosynthesis at 680 and 625

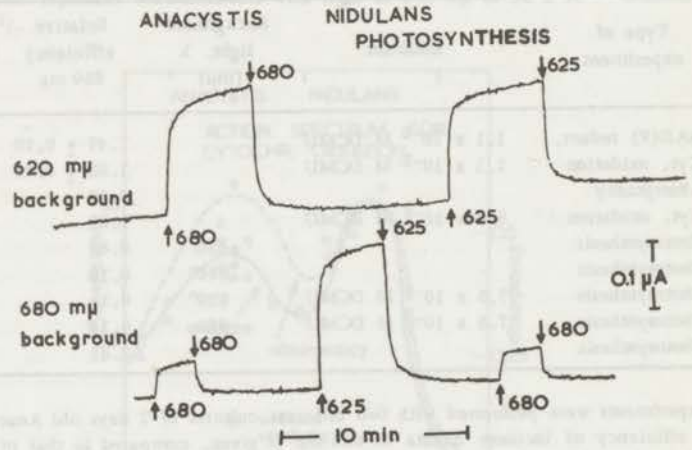

Fig. 4.8 Polarograph tracings of the rate of photosynthesis of a layer of about 4 μ thickness of 2 days old Anacystis nidulans. The first tracing was recorded in the presence of a continuous background of light of 620 mμ of an intensity of 10^{-9} einstein/(sec cm^2), the bottom tracing with a background of 680 mμ of 3 x 10^{-9} einstein/(sec cm^2). During the intervals marked by upward and downward pointing arrows the algae were also illuminated by a second beam of wavelength 680 or 625 mμ of an intensity of 3.5 and 2.7 x 10^{-10} einstein/(sec cm^2) respectively.

mμ with different constant backgrounds. These recordings show that the activities of light at these wavelengths are quite different for different colors of background light. These effects are, of course, the same as those which have usually been measured and plotted as "Emerson enhancement effects": the Emerson effect can be explained by the assumption that the products made in excess by system 1 at one wavelength react with the products made in excess by system 2 upon simultaneous illumination with light of a second wavelength (81). Thus the light energy which is wasted by the excess absorption in one or the other system, when either beam is applied separately, is used for photosynthesis upon combination of these beams.

Rows 6-8 of Table 4.2 show that the ratios of efficiencies for light of 625 and 680 mμ, obtained in three different ways under conditions at which according to the hypothesis system 2 determined these efficiencies, agree well. The rate of photosynthesis, which for unpoisoned algae is strongly dependent upon the kind of background light, is virtually independent of the background employed for DCMU poisoned algae, which is also in agreement with our hypothesis. However, the ratios given in rows 4 and 5 are somewhat different. Possible explanations of this discrepancy, which was also noted for Porphyridium (82), will be discussed below. Whatever will be the cause of the discrepancy, experiments such as those given by rows 1 and 2 strongly indicate that the reduction of NAD(P) is caused by the same photochemical system as that which causes cytochrome oxidation. This system, system 1, clearly can be distinguished from the DCMU-sensitive system 2.

4.3.5 Quantum requirement for NAD(P) reduction and cytochrome oxidation

As discussed (§ 3.3.1) the fluorometric method, in contradistinction to the absorptiometric method, cannot be used for a reliable measurement of the quantum requirement of coenzyme reduction, since the fluorescence yield of the possibly enzyme-bound $NAD(P)H_2$ is not known. The shape of the fluorescence action spectrum of $NAD(P)H_2$ (205) indicates that in Anacystis the absorption maximum of $NAD(P)H_2$ is not much shifted by binding to cell constituents. Earlier attempts to correlate absorption changes in the near ultra violet with the reduction of coenzyme were inconclusive, because it turned out that there were other substances (e.g. cytochrome) which contributed to the light-induced absorption changes in this region.

From the experiments in the preceding sections it followed that upon illumination with light of 620 mμ of not too high intensity, the photooxidation of cytochrome in the absence of DCMU is very small, so that for actinic light of 620 mμ little interfering absorption changes in the ultra violet from cytochrome reactions were to be expected. Fig. 4.9 shows the spectra of the steady-state absorption changes in the near ultra violet region.

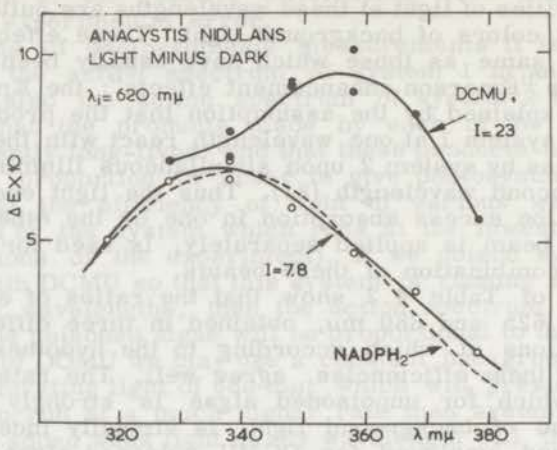

Fig. 4.9 Difference spectrum (light minus dark) of absorption changes in the ultra violet region in 2 days old Anacystis nidulans upon illumination. The 1.5% algal suspension was contained in a 1 mm cuvette. Points represented by open circles were obtained upon illumination with light of 620 mμ of an incident intensity of 7.8 x 10^{-10} einstein/(sec cm^2). Solid circles represent measurements in the presence of 4.3 x 10^{-5} M DCMU at a light intensity of 23 x 10^{-10} einstein/(sec cm^2). The dashed line gives, on an arbitrary scale, the absorption spectrum of $NADPH_2$ (212).

The first spectrum, obtained upon low intensity illumination of 620 mμ, closely resembles that of $NADH_2$ or $NADPH_2$, while the second one, obtained in the presence of DCMU, shows an additional increase around 360 mμ, presumably caused by a simultaneous oxidation of cytochrome. This is in accordance with earlier findings that cytochrome *f* (62), *c* (158) and the algal cytochrome 553 (154) show only a slight absorption difference between the oxidized and the reduced state around 340 mμ, but a considerable one at higher wavelengths. Furthermore, the kinetics of the ultra violet absorption changes upon illumination with light of 620 mμ were found to be the same as the kinetics of the changes in blue fluorescence. These results indicate that the absorption change at 340 mμ is mainly due to NAD or NADP, presumably also at higher actinic intensities and in the presence of DCMU.

From the initial rate of change in absorbancy, quantum requirements for coenzyme reduction and cytochrome oxidation were calculated, in the same way as in § 3.3.3 and 3.3.6. The difference in molar absorption coefficients between oxidized and reduced cytochrome was assumed to be 70 at 420 and 42 cm^{-1} mM^{-1} at 417 mμ (cf. ref. 154). Corrections for optical effects were applied as discussed in § 4.2. Quantum requirements measured for three different cultures are shown in Table 4.3. The low requirements obtained indicate that both NAD(P) re-

TABLE 4.3

Quantum requirements of light-induced NAD(P) reduction and of cytochrome oxidation in Anacystis nidulans.

Culture	Reactant	Actinic light		$1/\phi$, hν/eq.
		λ, mμ	intens.	
1	NAD(P)	620	7.8	2.1
1	NAD(P)	620	23	2.8
2	NAD(P)	620	3.9	2.4
2	NAD(P)	620	7.8	2.5
2	NAD(P)	620	23	2.6
3	NAD(P)	620	7.8	3.8
3	NAD(P)	620	15.6	2.8
1	cyt.	680	5.4	9.3
1	cyt.	680	7.7	8.0
1	cyt.	680	23	10.8
1	cyt. a)	680	7.7	7.3
1	cyt. a)	680	23	10.2
1	cyt. b)	680	15.0	9.0
2	cyt.	680	1.9	9.1
2	cyt.	680	3.9	6.7

a) in the presence of 2.1×10^{-5} M DCMU
b) in the presence of 4.3×10^{-5} M DCMU

The measurements were done with a 1.5% suspension of 2 days old Anacystis, contained in a 1 mm vessel, and taken from 3 different cultures. The light intensities, given in 10^{-10} einstein/(sec cm^2), are those at the place of the cuvettes. The quantum requirements, $1/\phi$, are calculated from the initial rate of absorption increase or decrease at 340 or around 420 mμ upon illumination and corrected for optical effects as described in § 4.2.

duction and cytochrome oxidation are important photochemical reactions in Anacystis. The implications of the values found and of the difference between the quantum requirements for coenzyme reduction and for cytochrome oxidation will be discussed below.

4.3.6 The fluorescence yield of $NAD(P)H_2$ *in vivo*

The fluorescence yield of $NAD(P)H_2$ produced in the light by Anacystis was measured in the same way as for Rhodospirillum rubrum (§ 3.3.4). The yield was found to be 1.9 times that of free $NADH_2$ or $NADPH_2$.

The experiments were done with a 1.4%, 2 days old suspension. The wavelength of actinic light was 620 mμ, the intensity 3.6×10^{-9} einstein/(sec cm^2). The measured absorbancy difference at 340 mμ was corrected as described in § 4.2. No correction was applied for the shift of the emission

spectrum of the photoreduced $NAD(P)H_2$ in the cells compared to that of free $NADH_2$ (75); this correction presumably is small. The correction for the absorption of excitation and fluorescence radiation in the cell was estimated to be + 6%.

4.3.7 Effect of CO_2

In order to examine whether light-induced coenzyme reduction also occurred under conditions of low CO_2 pressure, the kinetics of the blue fluorescence were also measured in algae grown and resuspended in a medium which was equilibrated with a mixture of air and 0.1% of CO_2, instead of 4%. $NaHCO_3$ was omitted from the culture medium and replaced by tris buffer at a concentration of 1 g/l. The medium was brought to pH 7.3 with HCl before sterilization. During growth the pH varied between 7.4 and 7.8; the culture was aerated with the mixture of air and CO_2 at a rate of about 200 ml/min.

We found that the growth rate of Anacystis was about the same under these conditions as under those normally applied. For suspensions of the same absorptancy at 680 mμ the amount of blue fluorescence in the dark and the difference between the steady-state fluorescence in actinic light of high intensity and in the dark were about twice as high for 2 days old Anacystis grown and measured at low CO_2 pressure as for algae grown and examined at a high partial pressure of CO_2. The initial rate of fluorescence increase per absorbed quantum was approximately the same.

These observations indicate that in algae grown under low CO_2 pressure the pool of photosynthetic NAD(P) is about twice as large, but that the efficiency for light-induced coenzyme reduction is approximately the same as for algae grown at 4% CO_2.

4.3.8 NAD(P) reduction in other blue-green algae

The increase of blue fluorescence upon illumination was also measured for two other species of blue-green algae. Fig. 4.10 shows recordings obtained with the filamentous blue-green alga Anabaena cylindrica and with a dense suspension (2.5%) of Schizothrix calcicola. For comparison a recording obtained with Anacystis is also shown, measured at the same intensity of fluorescence excitation and at the same sensitivity of the apparatus as for Schizothrix. In the experiments with Anabaena the sensitivity was 1.3 times lower.

The recordings show, that, although the levels of dark fluorescence vary strongly for the different species, the absolute amount and the initial rate of fluorescence increase upon illumination differ much less. This suggests that the efficiency for coenzyme reduction and the pool of photosynthetic NAD(P) are of the same order of magnitude in these algae. An exact comparison is not possible because the fluorescence yield of $NAD(P)H_2$ in Schizothrix and Anabaena is not known. If this

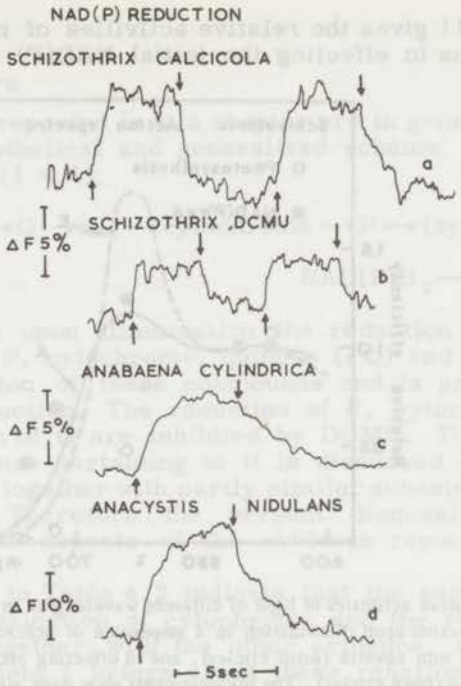

Fig. 4.10 Recordings of the blue fluorescence increase upon illumination and decrease upon darkening for a 2.5% suspension of 2 days old Schizothrix calcicola (curves (a) and (b)), a clump of filaments of Anabaena cylindrica (c) and for a 4% suspension of 3 days old Anacystis nidulans (d), contained in 1 mm quartz vessels. The fluorescence was excited by radiation of 340 mμ. The algae were illuminated with light of 680 mμ of an intensity of 3.5×10^{-10} einstein/(sec cm^2) (curves (a), (c) and (d)) or 7.0×10^{-10} einstein/(sec cm^2) (curve (b)). Curve (b) was obtained in the presence of 1.5×10^{-5} M DCMU. Further details are given in the text and in the legend of Fig. 4.2.

yield is the same for Schizothrix as for Anacystis, then the efficiency for NAD(P) reduction and the pool of photosynthetic NAD(P) are about equal for both species, since the ratio of absorbancies at 680, 340 (the wavelength of fluorescence excitation) and around 450 mμ are not very different for these algae. A comparison with Anabaena is more difficult, since the suspension was optically inhomogeneous because of clumping of the filaments.

DCMU diminished the difference between the steady-state level in the dark and the light for Schizothrix but had little effect on the initial rate of coenzyme reduction. With Anabaena no fluorescence increase upon illumination was observed in the presence of 1.5×10^{-5} M DCMU. Possibly part or all of the NAD(P) was in the reduced state already in the dark in the presence of inhibitor.

Fig. 4.11 gives the relative activities of red light of different wavelengths in effecting the initial NAD(P) reduction in Schizo-

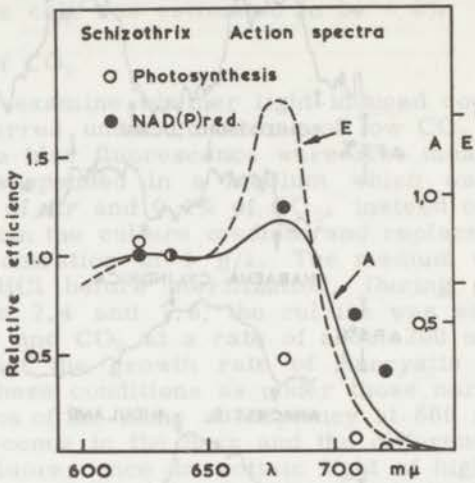

Fig. 4.11 Relative activities of light of different wavelengths in effecting the initial NAD(P) reduction upon illumination in a suspension of Schizothrix calcicola, contained in a 1 mm cuvette (solid circles), and in effecting photosynthesis in a thin layer of algae (open circles). The measurements were done with samples of different 1 day old cultures. The solid line gives the absorptancy of the suspension used for measuring NAD(P) reduction; the dashed line the absorbancy of a dilute suspension of the same algae as used in the photosynthesis experiments. The actinic light was filtered by means of heat-absorbing and reflecting filters and of Balzers B 40 interference filters, half-width 10 to 15 mμ. The other conditions were the same as in Fig. 4.10.

thrix. The results indicate about the same or a somewhat higher activity of chlorophyll *a* at 680 mμ than of phycocyanin at 620 mμ, and suggest a relatively high activity of light absorbed in the far red region (700 to 720 mμ). Because of the higher level of dark fluorescence and the corresponding higher noise level the precision was less than for the action spectrum of Anacystis (§ 4.3.2). For comparison the activity for photosynthesis was measured at the same wavelengths for a thin layer of Schizothrix taken from a suspension of the same age. As Fig. 4.11 shows, the relative activity of chlorophyll *a* at 680 mμ and in the far red region is markedly lower for photosynthesis than for coenzyme reduction. The algae were not evenly distributed on the electrode; the inhomogeneous distribution may have lowered somewhat the photosynthetic activity of strongly absorbed light compared to light of wavelengths where little absorption occurred. Because of this uncertainty no correction was applied for reflection at the platinum surface (see § 2.6).

4.4 *Discussion*

4.4.1 Action spectra

The experiments reported in this chapter are in general agreement with the hypothetical and generalized scheme, mentioned in the introduction (§ 4.1):

$$H_2O \rightarrow (\text{system 2}) \rightarrow Q \rightarrow PQ \rightarrow \text{cytochrome} \rightarrow P \rightarrow (\text{system 1}) \rightarrow NAD(P)H_2 \rightarrow CO_2$$

System 1 causes upon illumination the reduction of NAD(P) and the oxidation of P, cytochrome, quinone (PQ) and Q; system 2 causes a reduction of these compounds and is probably involved in O_2 production. The reduction of P, cytochrome and PQ and the oxidation of Q are inhibited by DCMU. This scheme and additional evidence pertaining to it is discussed extensively elsewhere (82, 84), together with partly similar schemes proposed by other authors. Therefore the present discussion will be restricted mainly to aspects of the evidence reported in this chapter.

The results given in Table 4.2 indicate that the same pigment system effects the oxidation of cytochrome and the reduction of coenzyme, and, together with the other evidence, support the hypothesis that system 1 brings about these reactions. This is also consistent with the experiments of others on NADP reduction by spinach chloroplasts (184, 247, 148, 120) and cellular extracts of Chlamydomonas reinhardi (182, see also ref. 181). Support is also given by the action spectra of Hoch and Martin (131a) and by the, probably more distorted, action spectra of Arnon *et al.* (19) for NADP reduction by chloroplasts.

As indicated by our action spectra, system 1 in young cultures of Anacystis nidulans shows an activity of chlorophyll *a* which is about equal to or somewhat higher than that of phycocyanin. System 2, as measured by the rate of photosynthesis in the presence of DCMU or with a background of light of 680 mμ, shows roughly six times higher activity for light absorbed by phycocyanin than by chlorophyll *a*. The experiments with Schizothrix calcicola yield similar results and indicate a roughly equal activity of system 1 in this alga for quanta absorbed by chlorophyll *a* at 680 mμ as for quanta absorbed by phycocyanin. The action spectrum of photosynthesis indicates that system 2 contains relatively little chlorophyll. The activity of phycoerythrin, a phycobilin which is also present in relatively high concentration in this alga, was not investigated because light of wavelengths lower than about 600 mμ interfered in the measurement of $NAD(P)H_2$ fluorescence. In Porphyridium cruentum system 1 contains about equal amounts of chlorophyll *a* and phycoerythrin and system 2 more phycoerythrin than chlorophyll *a* (81, 82).

The action spectra for photosynthesis of all blue-green algae

(with one exception (88)), and all red algae investigated (67, 123, 124, 45) are similar to those of Anacystis and Schizothrix in showing a lower efficiency of chlorophyll *a* than of the phycobilins. For the blue-green and red algae examined, light absorbed by chlorophyll *a* was also less active than that absorbed by the phycobilins in exciting chlorophyll *a* fluorescence (67). Further it was found that combination of light predominantly absorbed by chlorophyll *a* with light mainly absorbed by the phycobilins gave rise to enhancement of photosynthesis (Emerson effect) for several species of blue-green and red algae (40, 45, 82, 102, 191, 150). These observations indicate that system 1 contains relatively much chlorophyll *a* and system 2 relatively much phycocyanin and phycoerythrin in all these algae.

Kok and Hoch (167-169) studied kinetics of absorption changes around 700 mμ in various algae and in spinach chloroplasts. The results indicated that the absorption changes were caused by a pigment (P 700, P in the scheme) with an oxidation reduction potential E'_0 of 0.43V, which became reversibly bleached upon oxidation and is possibly a type of chlorophyll. A comparison with our results with those on P 700 changes in Anacystis nidulans (167, 169) adds to the evidence that system 1 is the same as the photochemical system responsible for the oxidation of P 700.

For Chlorella, the action spectra of photosynthesis (89) and of the Emerson enhancement effect (103, 118) indicate that the two systems contain different ratios of chlorophyll *a* types with different absorption maxima (see discussion in ref. 82). There are indications that Anacystis (122, 237) and other plants (174, 2) also contain different types of chlorophyll *a*, but our action spectra of Anacystis around 680 mμ are not sufficiently resolved to show a difference between the location of the chlorophyll *a* bands for system 1 and system 2. The action spectrum of Schizothrix (Fig. 4.11) indicates a relatively high content of a chlorophyll *a* type absorbing at long wavelengths for system 1 in this alga.

The fact that Emerson effects have been observed for various species of different groups of algae (91-93, 103, 118, 194, 40), for Elodea (137) and for *in vitro* reactions of higher plant chloroplasts (131, 120) indicates that in all green plants two photochemical systems with presumably similar functions exist. There are several indications that in photosynthetic bacteria only one system, similar to system 1, exists (cf. ref. 82): The action spectrum of bacteriochlorophyll fluorescence in Rhodopseudomonas spheroides is proportional to that of NAD(P) reduction (Chapter III) and to that of the photoinhibition of oxygen uptake (J.C. Goedheer and D.C. Fork, personal communication). Purple bacteria are unable to evolve oxygen, but, like in algae, light-induced spectral changes have been observed, due to the reduction of NAD(P) and the oxidation of cytochromes (Chapter III) and of a substance P 890, related to bacteriochlorophyll (67, 71, 10, 56, 251). The oxidation of P 890 is probably analogous

to the oxidation of P 700 in algae. Further the reactions of chromatophore preparations from purple bacteria are similar to those of chloroplasts in which system 2 has artificially been made inoperative, *viz.* "cyclic" photophosphorylation and the light-induced reduction of NAD and concomitant oxidation of dichlorophenol indophenol (200), cytochrome *c* (245, see also ref. 143) or of other substances with about the same or lower E'_0. A difference is that chloroplasts require the addition of redox compounds, such as FMN, for "cyclic phosphorylation". Finally, Blinks and van Niel (41) were unable to find an Emerson effect for photosynthesis of Rhodospirillum rubrum.

The photoreduction of CO_2 in the presence of H_2 by various green, blue-green and red algae (see ref. 230 for a recent review), which is not inhibited by DCMU (31) is probably a "bacterial-type" photosynthesis and only driven by system 1. This is indicated by the relatively high efficiency of far red light in Scenedesmus and Ankistrodesmus (33) and of red light in Porphyridium cruentum (L.N.M. Duysens, J. Amesz and J.M. van Dongen, unpublished experiments). It is possible that the light-induced assimilation of acetate by Chlamydobotrys, Chlorella vulgaris and some other algae (214, 264) is also effected by system 1 only.

There are a number of reports about the presence or absence of Emerson effects for various light-induced reactions of isolated chloroplasts (131, 120, 34, 25, 190). However, it appears that the results have as yet not given definite evidence about e.g. the way of reduction of the various Hill-oxidants. For this reason at this moment a discussion can better be postponed until more data become available.

Rabinowitch and Govindjee (93, 119) reported so-called negative Emerson effects (a lower rate of photosynthesis upon combination of two light beams than the sum of the rates obtained in each beam separately) for Anacystis nidulans and some other species. However, as Fig. 6 of ref. 93 suggested and as was later confirmed experimentally by Govindjee (119), the effect was caused by a non-linearity with light intensity of the apparent rate of photosynthesis in far red light rather than by an interaction of light of two different wavelengths. For this reason the term "negative Emerson effect" is confusing and not very appropriate; the "true" Emerson effect is caused by the interaction of two different pigment systems. Very recently Hoch *et al.* (132) presented direct and Jones and Myers (150) presented more indirect evidence that in Anacystis the effect was caused by an inhibition of oxygen uptake by far red light. The inhibition was saturated at much lower intensity than the rate of photosynthesis. The opposite effect had been noted (105) for Porphyridium cruentum. Our action spectra of photosynthesis (Fig. 4.1) were measured at higher intensity and are only little affected by the inhibition of oxygen uptake.

Some of our observations also indicate that the scheme discussed is too simple. According to the hypothesis the action

spectrum of photosynthesis of Anacystis measured against a strong background of light of about 620 mμ should be exactly proportional to that of cytochrome oxidation or coenzyme reduction in the presence of DCMU, since both action spectra should be proportional to the action spectrum of system 1. However, according to Table 4.2, there is a difference. An analogous discrepancy was observed in Porphyridium cruentum (82). Possible causes of this effect have been discussed elsewhere (82). The discrepancy may be due to an inhomogeneity of the algal suspension, which might be overcome by the use of a synchronized culture (138). Another explanation might be that the background illumination applied for the photosynthesis measurements, which lasted for 10 min or more, changed the distribution of the pigments for the two systems. One might also postulate the existence of a third system, which contains relatively much chlorophyll and is able to reduce NAD(P) and to oxidize cytochrome, but for some reason is not active in photosynthesis. However, more experimental evidence is needed, before these and other speculations become profitable.

Also the variation with wavelength of the saturation rate of photosynthesis for some algae (192, 119) and of the reduction of NADP by chloroplasts (95, 131a) is not explained by the simple scheme. The effect might be caused by a partial inactivation of the photochemical apparatus by strong excessive excitation of one of the two pigment systems. Variation of the saturation rate was not observed for the Hill reaction of oat chloroplasts with 2,6-dichlorophenol indophenol (25), which process is possibly driven by system 2 only.

On the whole, however, the experiments reported here and most other evidence known to us, may be satisfactorily described by the scheme discussed; it allows a quantitative interpretation of various action spectra which in general appears satisfactory.

4.4.2 Reactions of photosynthetic intermediates

The initial rate of coenzyme reduction upon illumination in Anacystis is not inhibited by a relatively high concentration of DCMU: this indicates that the primary photochemical reaction, responsible for NAD(P) reduction, is not inhibited and is consistent with the hypothesis that NAD(P) is reduced by system 1. The same applies to the experiments of Vernon and Zaugg (247) and of Jagendorf and Margulies (148), who reported that the light-induced reduction of NADP by a preparation of spinach chloroplasts to which di- or trichlorophenol indophenol and ascorbate were added was not inhibited by DCMU or CMU. Under these conditions no oxygen is evolved, and reduced chlorophenol indophenol is presumably oxidized by system 1 (cf. refs. 184 and 131a).

The rate of reoxidation of NAD(P)H_2 upon darkening after a period of illumination with moderate intensities of light of 620 or 630 mμ is approximately the same as the rate of reduction

upon onset of illumination. This indicates that the steady-state rate of photoreduction in light of 620 mμ is about the same as the initial rate. Upon illumination with light of 680 mμ, however, the steady-state rate is appreciably lower than the initial rate: light quanta of 680 mμ appear to be more active in effecting the initial reduction, but considerably less active in maintaining the steady-state reduction than quanta of 620 mμ. A similar effect is brought about by DCMU, which lowers the steady-state rate, but does not inhibit the initial reduction rate of NAD(P). These results suggest that the steady-state rate of coenzyme reduction is relatively low under conditions where system 2 is inhibited or is running at a low rate. The kinetics of coenzyme reduction (a few examples are given in Fig. 4.2) indicated that this lower rate of the steady-state photoreduction of NAD(P) was, under the conditions mentioned, not accompanied by a significantly lower steady-state level of $NAD(P)H_2$ concentration in the light. This suggests that the rate of dark oxidation also depends upon other photoproducts (perhaps ATP), for the generation of which system 2 or the cooperation of both systems is needed.

For Schizothrix calcicola the time courses of the blue fluorescence indicate that the initial rate of NAD(P) reduction is not inhibited by DCMU, but that the steady-state deflection of fluorescence is smaller in the presence of inhibitor. In Anabaena cylindrica the fluorescence increase upon illumination was inhibited by DCMU. However, the experiments with the other algae indicate that this is probably not due to a direct inhibition of the photochemical reduction. Possibly NAD(P) was in the reduced state already in the dark.

The time courses of coenzyme reduction in Anacystis indicate that, after a few or more seconds of illumination, the reoxidation of reduced coenzyme in the dark and consequently the steady-state rate of reduction in the light is only partially inhibited by DCMU. The degree of inhibition increased with light intensity. Since oxygen evolution is completely inhibited by a sufficiently high concentration of DCMU, the light-induced reduction of NAD(P) probably takes place at the expense of the oxidation of an intracellular hydrogen donor, which may be directly or indirectly regenerated by reduced coenzyme in a dark reaction. The same is indicated by the kinetics of the blue fluorescence of Schizothrix in the presence of DCMU. In Anacystis the pool of NAD(P) which can be reduced in the light is approximately 1/150 of that of chlorophyll *a* (on a molar basis).

The rapid reoxidation of $NAD(P)H_2$ in the dark may explain why Oh-hama and Miyachi (201) failed to observe a light-induced reduction of coenzyme in illuminated Chlorella ellipsoidea. In their experiments the time required to kill the algae following illumination may well have been sufficient for the reoxidation of the reduced coenzyme formed in the light. The changes in NAD(P) and $NAD(P)H_2$ concentrations reported (201, 202) are apparently due to relatively slow reactions, which

may be not directly connected to the photosynthetic process.

The enhanced fluorescence yield of photosynthetic $NAD(P)H_2$ in the cell indicates in agreement with the earlier observed shift of the fluorescence spectrum (75) a binding to cell constituents. This, however, does not necessarily indicate a binding to a photoreductase (see § 5.4) or related enzyme: the effects may also be caused by a non-specific binding to cell proteins (96, 21).

As mentioned in Chapter III it is not possible to obtain spectrophotometric evidence whether NAD or NADP is reduced in the intact cell. However, the results of *in vitro* experiments, among other things on coenzyme reduction in cellular extracts of green and red algae and higher plants (see Chapter V) and recently also of Anacystis and some other blue-green algae (36), indicate that in chlorophyll *a* containing organisms probably NADP is reduced in the light.

The kinetics of the absorption changes at 418 mμ indicate a reduction of oxidized cytochrome upon darkening in the presence of DCMU. Upon switching off the light after illumination with 680 mμ, the rate of reduction is roughly the same in the absence as in the presence of inhibitor. This indicates that the cytochrome is reduced also by substances other than the reduced photoproducts of system 2. The effect may be related to the incomplete inhibition of the reoxidation of $NAD(P)H_2$ in the dark: possibly oxidized cytochrome and $NAD(P)H_2$ react in a dark reaction. Enzymes catalyzing the reduction of cytochrome *c* by $NADPH_2$ have been obtained from spinach chloroplasts and from Chlorella pyrenoidosa (189, 236, 155). Chloroplast "transhydrogenase" (see Chapter V) catalyzes the reduction of cytochrome *f* by $NADPH_2$ (G. Forti, personal communication).

As mentioned, the difference spectrum of Anacystis upon illumination gives only indication for the reaction of one cytochrome in the light: a *c*- or *f*-type cytochrome with an α-band at about 554 and a γ-band at about 422 mμ (§ 4.3.3). The pool of oxidizable cytochrome is roughly 1/400 of that of chlorophyll *a*. Like in Porphyridium cruentum (82) no light-induced spectral changes were found which could be attributed to other cytochromes. Chance and coworkers (55) reported the light-induced oxidation of a *b*-type cytochrome in a pale-green mutant of Chlamydomonas upon illumination, and Lundegårdh (188) observed light-induced spectral changes of *b*-cytochromes in spinach chloroplasts upon high intensity illumination. On the other hand, Olson *et al.* (209), confirming our results, observed no light-induced oxidation of cytochrome b_6 in aerobic intact cells of Anacystis and Euglena, but only under conditions of anaerobiosis, in the presence of the inhibitor carbonyl cyanide *m*-chlorophenyl hydrazone or after disrupting of the cells. Bonner and Hill (41a) observed cytochrome b_6 in the difference spectrum of etiolated leaves, but after greening only cytochrome *f* was seen. It appears that from the experiments mentioned as yet no definite conclusions can be drawn about the function of cytochrome b_6 or other *b*-

type cytochromes, and that up to now no direct evidence has been reported about a participation of these cytochromes in photosynthesis of intact, rapidly photosynthesizing tissue. As will be discussed in § 4.4.3, the relatively low efficiency for the *f*-type cytochrome in Anacystis suggests a by-pass for this cytochrome in the photosynthetic chain. Forti *et al.* (ref. 98 and personal communication) observed a reduction, but no oxidation of added cytochrome *f* by illuminated chloroplasts, but the evidence indicates that the *in vitro* reduction is brought about by system 1 and not by system 2 as in our experiments (see also ref. 171). It is not yet clear if, and under which conditions, this reaction takes also place in the intact cell.

Summarizing, we may conclude that our knowledge about the role of cytochromes in photosynthesis is still far from complete and that more experimental evidence will be needed before definite statements about the function of these compounds can be given.

Lundegårdh (187, 188) interpreted his experiments on light-induced absorption changes in isolated chloroplasts as evidence against the scheme discussed in this chapter. To our opinion, especially since isolated chloroplasts may perform reactions which do not occur in the intact cell, there is insufficient reason for this statement. A detailed interpretation of the evidence is difficult because of lack of quantitative data, e.g. about the intensity and spectral distribution of the actinic light in these experiments.

4.4.3 Quantum requirements

Upon illumination, NAD(P) appears to be reduced in Anacystis with good efficiency. About 2.5 light quanta of 620 mμ are needed for the transfer of one electron or hydrogen. This number is not much different from that for NADP reduction by spinach chloroplasts (see Chapter V). As discussed, the kinetics indicate that the efficiency for coenzyme reduction remains approximately constant in light of 620 mμ and does not drop upon prolonged illumination as is the case with Rhodospirillum rubrum and Rhodopseudomonas spheroides (§ 3.3). The quantum requirement for NAD(P) reduction is in reasonable agreement with that for CO_2 reduction reported on a variety of different algae by a number of authors (90, 166). The experiments on blue fluorescence of Schizothrix and Anabaena indicate that the quantum requirement for coenzyme reduction in these algae is of the same order of magnitude as that in Anacystis. All these experiments are consistent with the conclusion that the reduction of CO_2 proceeds solely or mainly via $NAD(P)H_2$. The same is true for the quantum requirement in Chlorella vulgaris (between 2.1 and 8.5) reported earlier (76, 4).

Supporting evidence that NAD(P) is an intermediate in CO_2 reduction is the observation that $NADPH_2$ and ATP stimulate the fixation of CO_2 by spinach chloroplast preparations (239, 240).

The maximum rate of CO_2 fixation by isolated chloroplasts, supplied with soluble extracts is only about 10% of the rate observed in intact leaves; but the low rate may be due to dilution and loss of enzymes during preparation (226, 125). The reported absence of aldolase in Anacystis and some other blue-green algae (216, 217, 94) was interpreted as evidence (cf. ref. 152) that the Calvin-Benson scheme (Chapter I) has to be somewhat modified for these organisms. A completely different mechanism for CO_2 reduction than discussed by us was proposed by Warburg and coworkers (252-254), which, however, is not supported by the evidence discussed in this and the following chapter. Warburg's scheme will require modification anyhow, since a quantum requirement for photosynthesis of less than 4 (255) is thermodynamically improbable, if not impossible (76, 78).

Since in Anacystis at 620 mμ only part of the light is absorbed by system 1 (probably more quanta are absorbed at this wavelength by system 2 than by system 1), the number of quanta absorbed by system 1 needed for the reduction of one equivalent of NAD(P) is probably considerably lower than 2.5, perhaps 1. If system 2 requires also one light quantum for each electron transferred, then at least 8 quanta are needed for the reduction of one molecule of CO_2 and the production of one molecule of O_2. These 8 quanta will suffice, if ATP is generated by a reaction coupled with photochemical hydrogen transfer, rather than by a "cyclic" phosphorylation. Cyclic photophosphorylation in the presence of added cofactors, has been reported in preparations from chloroplasts of spinach (13, 14, 261, 146, 147, 149, 121) and other higher plants (262); non-cyclic phosphorylation in chloroplasts, dependent upon the light-induced reduction of NADP or of Hill-oxidants and the simultaneous evolution of oxygen or oxidation of dichlorophenol indophenol was also reported (16-18, 184, 161, 24). However, non-cyclic phosphorylation *in vitro* produces only one ATP per two reduced equivalents, a quantity which is probably insufficient for CO_2 reduction. There is insufficient evidence whether non-cyclic phosphorylation is more efficient in the living cell, or whether *in vivo* part of the ATP is produced by a non-cyclic reaction. In the absence of cofactors the rate of cyclic phosphorylation by isolated chloroplasts is very small (97). Recently chloroplast constituents have been reported as "natural cofactors" of cyclic phosphorylation (37, 235), but for one of these the optimal concentration needed is high; for the other it is not known. Forti and Parisi (99) observed a production of ATP in illuminated intact leaves of Spinacia and Saxifraga in the presence of CMU, but it is still uncertain if the rate of this reaction is comparable to that of photosynthesis. A minimum quantum requirement of 8 for photosynthesis or a little more, depending upon the way of ATP synthesis, is rather high compared to the experimentally determined quantum requirements. Mainly for this reason it was hypothesized that system 2 would need only one quantum for two electrons transported (170, 29). On the other hand, the

lowest quantum requirements for oxygen production which are reported by all authors (except Warburg and coworkers) are not much lower than 8 (90, ref. 112, p. 114) and according to a recent critical review (166) the "true" requirement is probably 8 or higher.

The quantum requirement for cytochrome oxidation in Anacystis upon illumination with light of 680 mμ was found, both in the absence and in the presence of DCMU, to be about 7 to 10 quanta per oxidized cytochrome molecule or equivalent. Although this number indicates that the photochemical oxidation and reduction of the cytochrome are important photosynthetic reactions, the efficiency is appreciably lower than that of coenzyme reduction. At lower intensities than those given in Table 4.3, the initial rate of cytochrome oxidation indicated an even higher quantum requirement. Since the efficiency for cytochrome oxidation for light of 620 mμ in the presence of DCMU is about 1.4 times lower than for light of 680 mμ, the efficiency for coenzyme reduction is roughly 5 times higher than for cytochrome oxidation. This suggests that only part of the electrons going from system 2 to system 1 are transported by the cytochrome. It must, however, be remarked that the calculation of the quantum requirements presented is based upon the assumption that, in accordance with our hypothesis, no light-induced reduction of cytochrome occurs immediately after onset of illumination in the presence of DCMU. This is not inconsistent with the dark reduction of oxidized cytochrome observed after shutting off the light, which runs at a rather low rate (Fig. 4.5), even after a large proportion of cytochrome has become oxidized. The relatively high quantum requirement (about 4.5) and the complicated and varying kinetics of the cytochrome in Porphyridium suggested a by-pass for the cytochrome in this species too (80).

CHAPTER V

SPINACH CHLOROPLASTS

5.1 *Introduction*

In this chapter results are given of experiments on the light-induced reduction of NADP and NAD by isolated spinach chloroplasts. We found the increase in blue fluorescence upon illumination of intact leaves of various higher plants (Spinacia oleracea, Centaurea cyanus, and the aquatic plants Vallisneria sp., Cabomba sp., Hydrilla verticillata and Hygrophila polysperma) to be too small for the study of the reduction of coenzyme in these tissues *in vivo* with sufficient precision. This may be due to the possibly low concentration of NADP in the chloroplasts of higher plants (see refs. 176, 61) and to the strong scattering of light in these tissues.

The results with isolated chloroplasts reported in this chapter indicate that the reduction of added NADP by an illuminated chloroplast suspension proceeds with high efficiency. A quantum requirement of about 6 per reduced NADP molecule was found. At high intensity of illumination the rate of reduction of added NAD was markedly lower than of NADP, but the results indicated at very low intensity about the same quantum requirement as for NADP reduction. Some experiments are described which yield evidence about the properties of the enzymes concerned in the reduction of both coenzymes.

5.2 *Materials and Methods*

"Broken chloroplasts" were prepared by a method essentially similar to that of Arnon *et al.* (15). 15-30 g of spinach leaves (*Spinacia oleracea* L., obtained from local greengrocers) were ground in a mortar with sand and 5-10 ml of a solution of pH 8.2, containing 3.5×10^{-1} M NaCl, 1.3×10^{-2} M tris buffer, 10^{-2} M ascorbate, and 6×10^{-4} M $MgCl_2$. The brei was filtered through nylon tissue, and the filtrate was centrifuged for 10 min at 1000 x g. The sediment of chloroplasts was resuspended in the above-mentioned solution and centrifuged again. Distilled water, usually 2 to 4 ml, was added to the sediment to give a suspension, containing about 1 to 2 mg chlorophyll per ml. Part of this suspension, in the following to be referred to as "chloroplast suspension", was centrifuged again for 15 min at 1000 x g to give a pale green supernatant, in the following to be referred to as "chloroplast extract". During all manipulations the temperature was kept at a few degrees C. Chloroplast suspension and extract were stored at 3°C before

use; the average time of storage was 1-1½ h. The chlorophyll concentration was measured by the method of Arnon (11).

Unless otherwise indicated the reaction mixture contained in a final volume of 0.3 ml: 0.04 ml chloroplast suspension, 0.08 ml chloroplast extract, 10^{-2} M NaCl, 10^{-1} M tris buffer, pH 7.9, 1.3 x 10^{-4} M $MgCl_2$ and 8 x 10^{-3} M ascorbate. The NADP and NAD concentrations will be specified separately. NAD, $NADH_2$ and NADP were purchased from Boehringer, Mannheim.

Absorption changes at 350 mμ upon illumination with red light were measured by means of the modified Zeiss PMQ II spectrophotometer (Fig. 2.5). The reaction mixture was contained in a 1 mm quartz absorption vessel. The actinic light was filtered by means of one of the following combinations of filters:
F1: water, 2 cm, Balzers Calflex AV 690 and Schott RG 1, 2 mm, which provided a broad band of high intensity, and transmitted 2.8% at 600 mμ, 70% at 680 mμ and 4.7% at 710 mμ.
F2: a combination of F1 and a Balzers B 40 interference filter, which transmitted a band with a maximum at 668 mμ and a half-width of 12 mμ.
F3: a combination of F1 and a Schott AL interference filter. The maximum of transmittancy was at 672 mμ, the half-width 19 mμ.

The light intensities given are the incident intensities at the place of the cuvette.

Upon illumination of a chloroplast suspension to which NADP or NAD was added, the absorbancy at 350 mμ increased steadily. The difference spectrum of the increase in absorbancy upon illumination when NADP was added was in good approximation the same as that of $NADPH_2$. Without NADP or NAD no increase was observed. This indicates that the absorption increase was caused by the reduction of these coenzymes. It was found that the increase in absorbancy caused by addition of a known amount of $NADH_2$ was approximately equal to that caused by addition of the same amount of $NADH_2$ to the buffer solution without chloroplasts Beer's law was valid with sufficient approximation in the range of concentrations used in our experiments.

In the following experiments the increase in absorbancy of the suspension occurring in the first 15 or 30 sec of illumination was taken as a measure of the initial rate of NADP or NAD reduction. A molar extinction coefficient of 5.56 cm^{-1} mM^{-1} at 350 mμ was used for $NADH_2$ and $NADPH_2$ (139, 212). All experiments were carried out at room temperature (approx. 22°C).

The absorptancy of the suspension in the region 660-690 mμ was measured with opal glass and corrected for scattering, as described in § 2.3, by subtracting the absorbancy at 740 mμ from that at other wavelengths.

5.3 *Results and Interpretation*

5.3.1 pH, concentration and light dependence

The pH of the reaction mixture, which contained tris at a concentration of 4 x 10^{-2} M and 1.9 x 10^{-3} M NADP or NAD, was varied by adding various amounts of HCl or NaOH to the buffer solution used for dilution of the chloroplast suspension and extract. The actinic light was filtered by means of F1; the intensity was 8.1 x 10^{-8} einstein/(sec cm^2), which was several times above light saturation. Under these conditions the rate of NAD reduction was found to be about 2.5 times smaller than that of NADP reduction; the optimal pH for both reactions was about 8.0. This value is higher than that reported for NADP reduction by San Pietro and Lang (222) and Wessels (260), who reported values of 6.9 and 7.5 respectively. The difference may be caused by the different experimental conditions, e.g. the different concentration of chloroplasts and enzymes.

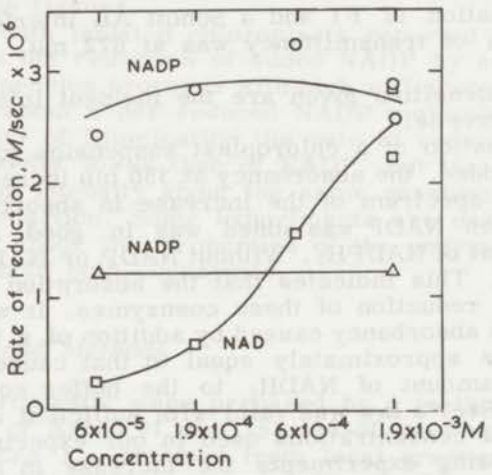

Fig. 5.1 The initial rate of NADP and NAD reduction by illuminated spinach chloroplasts as a function of concentration (on a logarithmic scale) of added coenzyme. Circles and squares represent measurements at an incident intensity of red light of 8 x 10^{-8} einstein/(sec cm^2) (filter F1), points represented by triangles were measured at 1.7 x 10^{-9} einstein/(sec cm^2) (filter F2, 668 mμ). The experiments with NAD were performed with a more active preparation than those with NADP. The other experimental conditions are given in the text.

Fig. 5.1 gives the initial rate of reduction of NADP and NAD as a function of concentration at two different light intensities. The figure shows that the rate of NADP reduction is virtually independent of concentration between 6 x 10^{-5} M and 1.9 x 10^{-3} M at high as well as at low light intensity, whereas the reduction rate of NAD varies strongly with the concentration. The saturat-

ing concentration of NADP is at least 30 times lower than of NAD.

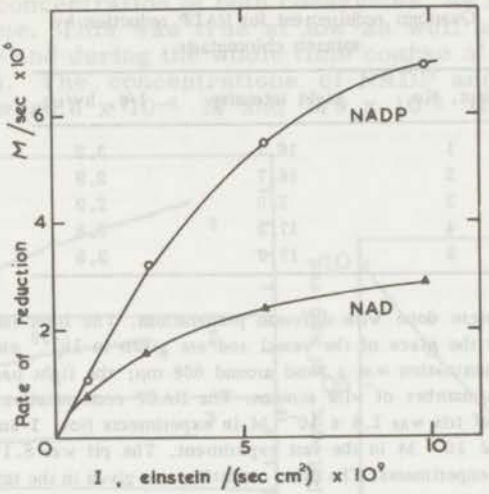

Fig. 5.2 Light dependency of NADP and NAD reduction by illuminated chloroplasts. The concentrations of NADP and NAD were 6×10^{-4} M. Illumination was a band around 672 mμ (filter F3). The other experimental conditions are given in the text.

The rate versus light intensity curves (Fig. 5.2) showed a lower intensity for saturation of NAD reduction than of NADP reduction. It can be seen that the rate of NAD reduction at high intensity is considerably lower than that of NADP; but at the lowest intensity measured there was much less difference between the rates of reduction of the two coenzymes. This suggests that at still lower intensities the rates of NAD and NADP reduction (too low to be measured with sufficient precision) are probably equal to each other.

5.3.2 Quantum requirements for NADP reduction

Table 5.1 shows the quantum requirement for NADP reduction, measured for a number of different preparations of suspensions of chloroplasts. The quantum requirement, $1/\phi$, is given as the number of light quanta, needed to bring about the reduction of one equivalent (half a molecule) of NADP, and was calculated in a similar way as in § 3.3.3. Corrections were applied for the obliqueness of the actinic beam and for reflections at the vessel walls and the opal glass. It was assumed that the opal glass reflected 50% of the light (223); since the suspensions had a high absorptancy (roughly 0.9), the latter correction was only about 5%. The initial rate of NADP reduction was calculated from the

TABLE 5.1

Quantum requirement for NADP reduction by spinach chloroplasts.

Expt. No.	Light intensity	$1/\phi$, hν/eq.
1	16.7	3.2
2	16.7	2.9
3	5.0	2.9
4	17.3	2.8
5	17.9	3.5

The measurements were done with different preparations. The light intensities are those of the actinic beam at the place of the vessel and are given in 10^{-10} einstein/(sec cm^2). The wavelength of illumination was a band around 668 mμ; the light passed through filter combination F2 and a number of wire screens. The NADP concentration was 1.9×10^{-3} M, the concentration of tris was 1.6×10^{-2} M in experiments Nos. 1 and 2, 4×10^{-2} M in Nos. 3 and 4, and 10^{-1} M in the last experiment. The pH was 8.1 in the first three and 7.9 in the last two experiments. The other conditions are given in the text.

increase of absorbancy during the first 15 sec of illumination. As Table 5.1 shows, the quantum requirement for NADP reduction was close to 3 quanta per equivalent. The average value was 3.05 with a standard error of $\pm$ 0.15.

5.3.3 Reduction in mixtures of NADP and NAD

The light-induced reduction of NADP has been found to proceed only in the presence of a soluble enzyme, photosynthetic pyridine nucleotide reductase (PPNR) (222), which was readily extractable from the chloroplast. PPNR was later found to be identical (64) to the cofactor for the light-induced reduction of methaemoglobin, earlier discovered by Hill and coworkers (63). The enzyme was recently renamed ferredoxin by Tagawa *et al.* (234) in view of its similarity to bacterial ferredoxins. It was found to contain iron and to be able to react in an oxidation reduction reaction (234, 156, 141, 108, 263, 54); the E_o' was reported to be -0.42 V (234). Recent experiments indicate the requirement of a second enzyme in NADP reduction, a flavoprotein (160, 234, 225, 65). Enzymes similar to ferredoxin and the flavoprotein were also obtained from Chlorella (114), Anacystis (228) and Nostoc (36). Ferredoxin was recently reported to stimulate cyclic photosynthetic phosphorylation, but only at relatively high concentration (235). Vernon (248) reported a light-induced reduction of NADP and NAD in the presence of ascorbate by an aqueous colloidal solution of chlorophyll to which ferredoxin was added.

In order to obtain information about the enzymes concerned in the reduction of NADP and NAD we did measurements on the rate of coenzyme reduction in preparations containing both

NAD and NADP. In all experiments it was found that the rate of coenzyme reduction was the same in the presence of a relatively high concentration of both coenzymes, as in the presence of NADP alone. This was true at low as well as at saturating light intensity and during the whole time course of the experiment (1 to 2 min). The concentrations of NADP and NAD in these experiments were 6×10^{-4} M and 1.9×10^{-3} M respectively.

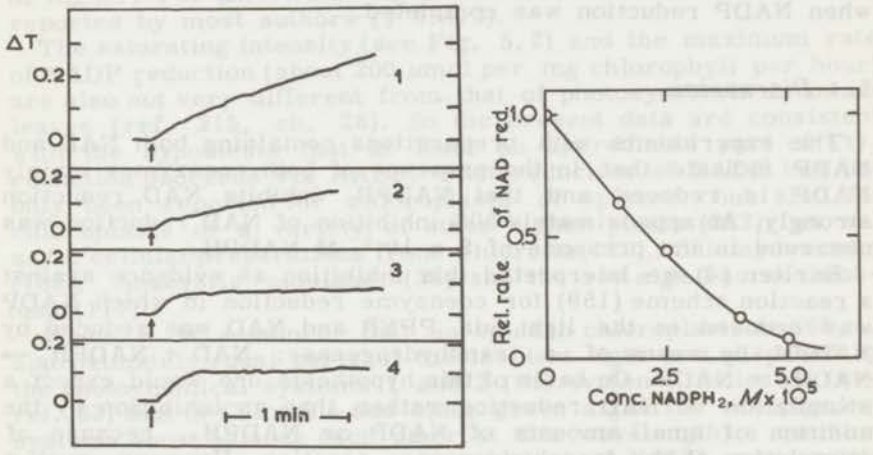

Fig. 5.3 Recordings of changes in transmittancy at 350 mμ of a suspension of illuminated chloroplasts in the presence of NAD and NADP. The relative transmittancy changes are given as fraction of the transmittancy at the beginning of the experiments. The NAD concentration was 1.9×10^{-3} M. Curve (1) was obtained when no NADP was added, curves (2), (3) and (4) when NADP was added to a final concentration of 3, 5 and 8×10^{-5} M, respectively. An upward pointing arrow indicates onset of actinic illumination; the intensity was 8×10^{-8} einstein/(sec cm^2) (filter F1).

Fig. 5.4 Inhibition by $NADPH_2$ of NAD reduction in illuminated chloroplasts. The reaction mixture contained, in a volume of 0.31 ml, 0.04 ml chloroplast suspension, 0.12 ml chloroplast extract, 1.5×10^{-3} M NAD, 8×10^{-3} M NaCl, 8×10^{-2} M tris, 10^{-4} M $MgCl_2$ and 6.5×10^{-3} M ascorbate. The concentration of chlorophyll a was 0.16 mg/ml. The light intensity was 7×10^{-8} einstein/(sec cm^2) (filter F1). Corrections varying between 0 and 15% were applied because control experiments indicated a small decrease of activity during the set of experiments.

Fig. 5.3 shows some typical recordings of absorption changes obtained upon illumination of chloroplast suspensions containing a high concentration of NAD and a low one of NADP. The recordings show that, when 5 or 8×10^{-5} M NADP was added, no more reduction of coenzyme was observed after the first 20 sec of illumination. The increase of absorbancy upon illumination approximately corresponded to the reduction of an amount of coenzyme which was the same as that of added NADP. This indicates that under these conditions only NADP was reduced. The

second recording shows that the reduction of NAD was slowed down, but not completely stopped when a lower concentration of NADP was added.

The above observations indicate that $NADPH_2$ strongly inhibits the reduction of NAD. Fig. 5.4 shows, for a different chloroplast preparation, the percentage inhibition of NAD reduction by $NADPH_2$. The percentage inhibition was calculated from the rate of absorbancy change, 45 sec after onset of illumination, when NADP reduction was completed.

5.4 *Discussion*

The experiments with preparations containing both NAD and NADP indicate that in the presence of both coenzymes mainly NADP is reduced and that $NADPH_2$ inhibits NAD reduction strongly. An approximately 50% inhibition of NAD reduction was observed in the presence of 2×10^{-5} M $NADPH_2$.

Earlier (4) we interpreted this inhibition as evidence against a reaction scheme (159) for coenzyme reduction in which NADP was reduced in the light via PPNR and NAD was reduced by $NADPH_2$ by means of a transhydrogenase: $NAD + NADPH_2 \rightarrow NADH_2 + NADP$. On basis of this hypothesis one would expect a stimulation of NAD reduction rather than an inhibition by the addition of small amounts of NADP or $NADPH_2$, because of stimulation of the transhydrogenase reaction. However, such a stimulation was neither observed by us nor by others (159, 61). Recently a modified scheme was proposed (160, 179), partly based on studies with a specific antibody for the transhydrogenase to be mentioned below. It was suggested that the reduction of free NADP and NAD occurs in two steps and that the reduction of both coenzymes is mediated by the same enzymes. By way of the photoreductase, bound NADP is reduced and subsequently bound $NADPH_2$ reduces free NADP or NAD. The latter reactions are catalyzed by the same transhydrogenase, a flavoprotein. The reaction scheme is similar to that proposed by Tagawa *et al.* (234, see also ref. 65), except that the latter authors postulated a flavoprotein catalyzed reduction of NADP by reduced ferredoxin instead of by bound $NADPH_2$. At present the evidence (54) favors the latter mechanism.

As Keister *et al.* (160) recognized, the above-mentioned reaction scheme is not inconsistent with the inhibition instead of stimulation of NAD reduction by $NADPH_2$: the effect can be explained by an inhibition of the flavoprotein catalyzed reduction of NAD.

The requirement of about 3 quanta per equivalent at 668 mμ shows that NADP is reduced with rather high photochemical efficiency. The change in Gibbs free energy of the reaction $NADP + H_2O \rightarrow NADPH_2 + \frac{1}{2}O_2$ is 26 kcal/equivalent of $NADPH_2$ formed at equal concentrations of NADP and $NADPH_2$ and an oxygen pressure of 0.2 atm. Since the energy of 3 einsteins amounts to 129 kcal at 668 mμ, 20% of the light energy is used

in coenzyme reduction. If in the plant all $NADPH_2$ is utilized for CO_2 reduction, and all CO_2 reduction proceeds via $NADPH_2$, a quantum requirement of 3 quanta per equivalent for NADP reduction in the living cell would correspond to a quantum requirement of 4 x 3 = 12 per molecule of CO_2 reduced in photosynthesis. This value is in fair agreement with the numbers reported for leaves of higher plants (257, 111, 87), which were about 11 or higher. For unicellular algae not much lower values have been reported by most authors (§ 4.4.3).

The saturating intensity (see Fig. 5.2) and the maximum rate of NADP reduction (about 200 μmol per mg chlorophyll per hour) are also not very different from that of photosynthesis in intact leaves (ref. 215, ch. 28). So the present data are consistent with the hypothesis that at least an appreciable part of CO_2 reduction proceeds via NADP. Photoreduction of NADP has not only been reported for chloroplasts of spinach, but also for chloroplasts of a variety of other higher plants (262), as wel as for cellular preparations from Chlorella (129), Chlamydomonas (182), Anacystis nidulans (36) and the red alga Laurencia obtusa (117).

When it is assumed that in spinach chloroplasts at 668 mμ approximately equal parts of the incident light are absorbed by the photochemical systems 1 and 2 (the evidence indicates (see ref. 82) that in Chlorella and other green algae the two pigment systems about balance each other at this wavelength) a quantum requirement of 3 per reduced equivalent of NADP corresponds to the transfer of one electron per 1.5 quanta for each system (cf. Chapter IV).

The quantum requirements for NADP reduction reported here are appreciably lower than the number of about 8 quanta per equivalent reported by San Pietro *et al.* (221). The reason for this discrepancy is not known. Black *et al.* (35) more recently found a number of about 4.5 at 675 mμ and very high values in regions of low absorption. Later (95) it was reported that these numbers were too high: when the absorption of the chloroplast suspension was measured by means of the light-diffusing plate technique (as in our experiments), a number of 2.6, which agrees well with our results, was found. The requirement was reported to be essentially independent of wavelength from 380 to 680 mμ. Also in agreement with our results are those of Yin *et al.* (268), who found a value of 3.2 for a broad band of red light and about the same or a somewhat higher efficiency for the Hill reaction with ferricyanide, and those of Hoch and Martin (131a), who very recently reported values of 2 and higher.

As Fig. 5.2 indicates, the quantum requirement and rate of reduction of NAD are probably the same as of NADP at low light intensity, but appreciably lower at higher intensities. The concentration of NAD needed to bring about a sizable rate of reduction is much higher than of NADP. This, and the inhibition of NAD reduction by $NADPH_2$ indicates that in chloroplasts of intact plants only NADP is reduced.

SUMMARY

The experiments reported here were performed in order to obtain quantitative evidence about the photosynthetic reactions of nicotinamide-dinucleotide in the living cell. Most experiments were done with intact unicellular photosynthetic organisms by means of sensitive spectrophotometry. The effect of various experimental parameters, such as the wavelength and intensity of photosynthetic illumination, on the intracellular rate of coenzyme reduction or oxidation was measured and compared to the rate of the oxidation of cytochromes and of oxygen evolution.

The first chapter gives a short introduction to the subject. The second deals with the experimental methods used in the present study. A description is given of the culturing of algae and bacteria, of apparatus for measuring changes in absorbancy and fluorescence of algal and bacterial suspensions occurring upon photosynthetically active illumination and of a polarograph for measuring photosynthetic O_2 production. Further a discussion is given of the optical effects caused by scattering of light by the suspensions and by the fact that the light-absorbing substances are contained within small particles and not homogeneously dispersed in solution. It is shown that the correction for the latter effect is relatively larger for a small absorbancy change than for the total absorbancy of the suspension.

In Chapter III the results are given of measurements on light-induced intracellular NAD(P) (NAD or NADP) reduction and cytochrome oxidation in the non-sulphur purple bacteria Rhodospirillum rubrum and Rhodopseudomonas spheroides. The reduction of NAD(P) was measured by means of the blue fluorescence and by means of the absorbancy at 340 mμ of NAD(P)H_2; the oxidation of cytochromes by means of the decreased absorbancy of the oxidized forms in the Soret region. The bacteria were grown and resuspended before measurement in various organic media. The kinetics of NAD(P) reduction upon illumination and oxidation upon subsequent darkening indicated that the rate of light-induced reduction of NAD(P) was not constant during illumination but passed through a maximum after a few seconds of illumination and afterwards gradually dropped to a much lower value. With a few exceptions the lowest quantum requirement for the reduction was found to be 2 to 3 quanta per hydrogen or electron transferred. On the other hand, the kinetics of the absorbancy changes at 420 and 428 mμ in Rhodospirillum indicated a high turn-over rate of *b*- and *c*-type cytochromes also during prolonged illumination. 2-heptyl-4-hydroxy-quinoline-N-oxide and fluoroacetate only partially inhibited the reduction of NAD(P). In Rhodopseudomonas, at a few selected wavelengths in the region of carotenoids and bacteriochlorophyll absorption, the relative activity of light in effecting NAD(P) reduction was proportional to that in effecting bacteriochlorophyll fluorescence.

This indicates that both light processes are brought about by the same pigment system. These results, together with those obtained by others, are discussed in § 3.4.

Chapter IV gives the results of measurements on light-induced NAD(P) and cytochrome reactions in blue-green algae. In Anacystis nidulans, illumination with red light caused an increase in blue fluorescence and an increase of the absorbancy around 340 mμ, which were caused, as indicated by the spectra of the fluorescence and the absorbancy increase, by the reduction of NAD or NAD(P). Upon subsequent darkening $NAD(P)H_2$ was re-oxidized. The absorbancy difference spectrum in the violet and green region indicated the oxidation of a *c*- or *f*-type cytochrome upon illumination. Action spectra of Anacystis nidulans were measured for the reduction of NAD(P), the oxidation of cytochrome and for photosynthesis. The results, discussed in §4.4, could be explained by means of the hypothesis that photosynthesis in green plants is brought about by 2 primary light reactions, operating in series and driven by two different pigment systems. Pigment system 1 in Anacystis contains about equal amounts of chlorophyll *a* and phycocyanin and causes upon illumination the reduction of CO_2 via NAD(P) and the oxidation of the cytochrome. System 2 contains relatively much phycocyanin and little chlorophyll *a* and causes, via intermediate substances, the reduction of the cytochrome with the concomitant evolution of O_2. Consistent with this was the efficiency for NAD(P) reduction (about 2.5 quanta per equivalent at 620 mμ); the efficiency for cytochrome oxidation is probably much lower, which indicates a by-pass for the cytochrome in the photosynthetic chain between the 2 systems. A few experiments with other blue-green algae yielded results, similar to those obtained with Anacystis.

The quantum requirement for the reduction of NADP by illuminated spinach chloroplasts was found to be about 3 quanta per equivalent (Chapter V). This is in good agreement with the quantum requirements for photosynthesis in intact plants and is consistent with the conclusion that $NADPH_2$ is an intermediate of CO_2 reduction in algae and higher plants. It was found that the light-induced reduction of NAD is strongly inhibited by $NADPH_2$. The effect is probably due to an inhibition of the flavoprotein enzyme which catalyzes the reduction of NAD and NADP.

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ABBREVIATIONS

ADP , adenosine diphosphate
ATP , adenosine triphosphate
CMU , *p*-chlorophenyl-1,1-dimethylurea
DCMU , 3-(3,4-dichlorophenyl)-1,1-dimethylurea
FMN , flavine-mononucleotide
$FMNH_2$, flavine-mononucleotide, reduced form
HOQNO , 2-heptyl-4-hydroxy-quinoline-N-oxide
NAD , nicotinamide-dinucleotide (diphosphopyridine-nucleotide)
$NADH_2$, nicotinamide-dinucleotide, reduced form
NADP , nicotinamide-dinucleotide phosphate (triphosphopyridine-nucleotide)
$NADPH_2$, nicotinamide-dinucleotide phosphate, reduced form
NAD(P) , NAD and/or NADP
$NAD(P)H_2$, $NADH_2$ and/or $NADPH_2$
PMS , phenazine methosulphate
tris , tris(hydroxymethyl)aminomethane

Op verzoek van de Faculteit der Wiskunde en Natuurwetenschappen volgt hier een kort overzicht van het verloop van mijn academische studie.

Na het behalen van het eindexamen Gymnasium B aan het Coornhert Gymnasium te Gouda in 1951, begon ik in september van dat jaar mijn studie in de scheikunde aan de Rijksuniversiteit te Utrecht.

Het candidaatsexamen (letter g) werd afgelegd in december 1954. Mijn verdere studie geschiedde onder leiding van de Hoogleraren Dr. J. Th. G. Overbeek, Dr. J. M. Bijvoet, wijlen Dr. F. Kögl, Dr. Ir. J. Smittenberg en van Dr. L. N. M. Duysens. Het doctoraal examen met hoofdvak scheikunde en bijvakken fysische scheikunde en biofysica werd afgelegd in april 1958. In mei 1958 begonnen mijn werkzaamheden op de afdeling Biofysica van het Kamerlingh Onnes laboratorium der Rijksuniversiteit te Leiden. Van mei 1958 tot december 1960 was ik in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO). In januari 1961 werd ik benoemd tot wetenschappelijk ambtenaar 1e klasse en in mei 1963 tot wetenschappelijk hoofdambtenaar bij de Biofysica.