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Photoreactions of flavin mononucleotide and a flavoprotein with zwitterionic buffers*

The flavin moiety, whether free or bound as in a flavoprotein, is potentially a reactive system if illuminated The anaerobic photoreduction of free flavin by either various nitrogen-containing compounds such as amino acids[1-3] and EDTA[3,4] or by intramolecular reaction with the ribityl side chain[5-7] has been described. It has also been demonstrated that nitrogen-containing compounds can be used to photoreduce the flavin moiety of various flavoproteins[3,8]. In this report the photoreduction of FMN and a flavoprotein by five zwitterionic buffers described by GOOD *et al*[9] has been investigated

FMN spectra were recorded with a Beckman DB spectrophotometer utilizing anaerobic Thunberg cuvettes. Actinic light of 1250 ft candles for the photoreduction was provided by two Cool White fluorescent bulbs. Percentage reduction of the flavin was calculated from absorbance measurements at 447 mμ

The five buffers tested for the anaerobic photoreduction of $1 \cdot 10^{-4}$ M FMN were *N*-tris-(hydroxymethyl)methylglycine (Tricine), *N*,*N*-bis-(2-hydroxyethyl)glycine (Bicine), *N*-tris-(hydroxymethyl)-2-aminoethanesulfonic acid (TES), 2-(*N*-morpholino)ethanesulfonic acid (MES) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) In addition the photoreduction of FMN in Tris, phosphate and pyrophosphate buffers was studied. As shown in Fig. 1, the buffers can be placed in three categories according to the rate at which they support photoreduction Tricine and Bicine both react rapidly with FMN TES and MES react somewhat more slowly, while in the cases of Tris, phosphate, pyrophosphate and HEPES, the photoreduction is relatively slow

The absorption spectra of FMN at various times after the onset of photoreduction utilizing Tricine as the electron donor are shown in Fig. 2. The reduction was over 95 % complete after 60 sec of illumination. No semiquinone formation was detected as indicated by the lack of increase in absorbance in the 600-mμ region at intermediate stages of reduction. The reduced flavin was completely oxidized by the admission of oxygen to the system. The presence of clearly defined isosbestic points and the superposition of the initial and final spectra after the admission of oxygen indicate a lack of degradation of the flavin during the course of photoreduction. Similar spectra were obtained utilizing MES, TES and HEPES as the electron donor except that the time required to attain the same degree of reduction increased

In contrast to the photoreduction with the zwitterionic buffers, the reduction of FMN in Tris, phosphate and pyrophosphate was slow, and the spectra obtained were different. In the cases of these buffers, a shoulder appeared in the 420-mμ region in the intermediate stages of reduction, and the original oxidized FMN spectrum was not obtained upon the admission of oxygen to the system. The spectral changes were taken to be evidence for the irreversible degradation of the flavin caused by intramolecular reduction of the isoalloxazine ring by the ribityl side chain[5]. Although

Abbreviations Tricine, *N*-tris-(hydroxymethyl)methylglycine, Bicine, *N*,*N*-bis-(2-hydroxyethyl)glycine, TES, *N*-tris-(hydroxymethyl)-2-aminoethanesulfonic acid, MES, 2-(*N*-morpholino)-ethanesulfonic acid, HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

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HEPES buffer supported a similar rate of photoreduction as Tris, phosphate or pyrophosphate, it appeared to protect the flavin against the possibility of intramolecular reduction and subsequent degradation.

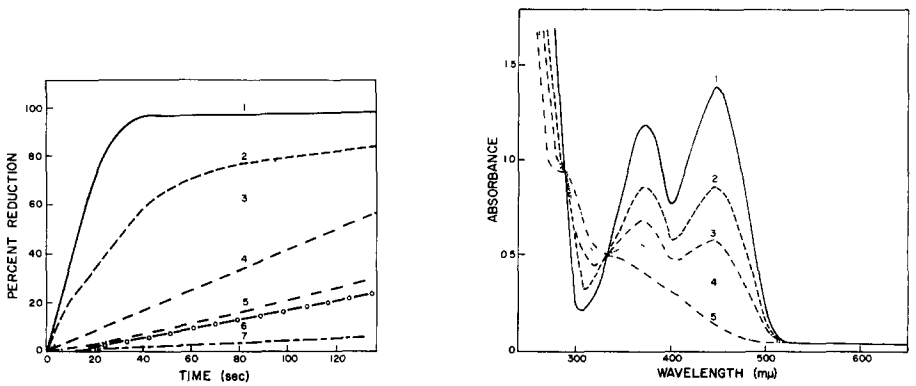

Fig 1 Rate of FMN photoreduction with various buffers The following buffers at a final concentration of $6.67 \cdot 10^{-2}$ M and pH 7.0 were mixed with FMN (final concn $1 \cdot 10^{-4}$ M) in the dark and then irradiated with 1250 ft candles of white light Curve 1, Tricine or Bicine; Curve 2, TES, Curve 3, MES, Curve 4, Tris; Curve 5, phosphate; Curve 6, HEPES, Curve 7, pyrophosphate (pH 8.3)

Fig 2 Anaerobic FMN spectra in Tricine buffer Final concentration of FMN was $1 \cdot 10^{-4}$ M, and the buffer at pH 7.0 was $6.67 \cdot 10^{-2}$ M For increasing total illumination times· Curve 1, zero sec, Curve 2, 10 sec, Curve 3, 15 sec, Curve 4, 25 sec, Curve 5, 120 sec. An identical spectrum with Curve 1 was reobtained upon admission of air after illumination was completed

Photoreduction of the flavoprotein glucose oxidase with Tricine is shown in Fig. 3 In contrast to the free FMN photoreduction, formation of semiquinone was observed in the 550–600- and 320-mμ regions of the spectra. MASSEY AND PALMER[8] have demonstrated the occurrence of two distinct forms of glucose oxidase semiquinone, depending upon the pH employed. Since the pH of transition between the two forms is approx. 7.5, it appears likely that the spectra shown in Fig 3 represent a mixture of the two forms. Upon admission of oxygen to the cuvette, the spectrum of the fully oxidized enzyme was obtained, indicating the changes to be reversible.

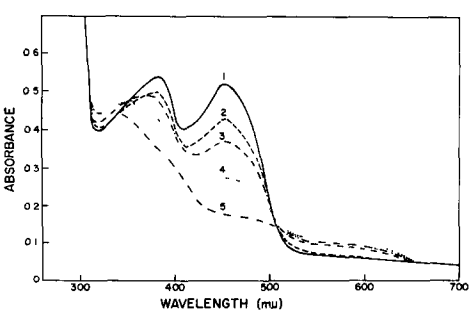

Fig 3 Anaerobic glucose oxidase spectra with Tricine Experimental conditions similar to those for Fig 1. At zero time 12.5 mg glucose oxidase in 0.5 ml phosphate buffer at pH 7.0 was added to 2.5 ml of Tricine at pH 7.0 so that the final buffer concentration was $6.67 \cdot 10^{-2}$ M For increasing illumination time. Curve 1, zero time, Curve 2, 5 min; Curve 3, 10 min, Curve 4, 20 min; Curve 5, 80 min An identical spectrum with Curve 1 was reobtained upon admission of air for 2 min.

Photoreduction of FMN or flavoprotein in the presence of the zwitterionic buffers described by GOOD *et al.*[9] should be of interest and concern to those using these or similar buffers. The utilization of Tricine and similar buffers is becoming more prevalent. When these buffers are employed with flavins or flavoproteins under normal laboratory light conditions, one may expect flavin reduction Aerobic conditions will lead to H_2O_2 production and decomposition of the buffer. Anaerobic conditions will lead to net reduction of the flavin unless illumination is excluded. Such reduction may lead to anomalous results in kinetic studies Presence of redox dyes in a flavin buffer system will lead to reduction of the dye An FMN–Tricine system was found to give rapid reduction of 2,6-dichlorophenolindophenol at light intensities normally encountered in the laboratory. In this case the dye served as the terminal electron acceptor.

This study has established that zwitterionic buffers such as Tricine can be utilized like EDTA for the production of flavoprotein semiquinone. An advantage of using Tricine in place of EDTA might be that Tricine would not bind metal ions to the same extent as EDTA. Thus the use of Tricine might be helpful in the study of metalloflavoproteins

The low reactivity of HEPES with flavins should be emphasized Although HEPES supported the same low rate of photoreduction as did phosphate, it was apparent from the spectral data that HEPES protected the FMN from the irreversible degradation evident in the case of phosphate. Thus in the pH range from 7.0 to 8.0, HEPES would appear to be the preferable buffer in illuminated biological systems. Protection against photoreduction of flavin may be the reason why HEPES is so effective in supporting high rates of photosynthesis in isolated chloroplasts[10].

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