Photosensitized NAD(P)H Regeneration Systems; Application in the Reduction of Butan-2-one, Pyruvic, and Acetoacetic Acids and in the Reductive Amination of Pyruvic and Oxoglutaric Acid to Amino Acid [1]

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The photosensitized formation of NAD(P)H by enzyme-catalysed processes has been accomplished. With $Ru(bpy)_3^{2+}$ as sensitizer, methyl viologen, MV^{2+} as primary electron acceptor, and $(NH_4)_3EDTA$ or 2-mercaptoethanol, NADPH is formed in the presence of ferredoxin $NADP^+$-reductase as enzyme catalyst. Zinc(II)meso-tetramethylpyridiniumporphyrin, $ZnTMPyP^{4+}$ is used as sensitizer for the photoinduced production of NADH using the same components and lipoamide dehydrogenase as enzyme catalyst. The photoinduced NAD(P)H regeneration systems have been coupled to secondary enzyme-catalysed processes such as the reduction of butan-2-one to butan-2-ol, pyruvic acid to lactic acid, acetoacetic acid to β-hydroxybutyric acid, as well as to the reductive amination of pyruvic acid to alanine and of α-oxoglutaric acid to glutamic acid. The products exhibit high optical purity and the enzymes and the coenzymes show high turnover numbers and stability.

Reduced nicotinamide cofactors (NADH or NADPH) play an important role in a variety of enzyme-catalysed reactions.[2] Application of these reactions in biotechnological processes which result in valuable chemicals requires effective means for the regeneration of NAD(P)H. Unfortunately, the use of electrochemical means or powerful reducing agents leads to non-selective reduction of the nicotinamide cofactor and consequently to poor turnover numbers and efficiencies.[3] Several effective chemical and electrochemical systems for the regeneration of NAD(P)H have been reported in recent years by Whitesides and his co-workers.[4–6] These include enzyme-catalysed reduction of $NAD(P)^+$ to NADPH, *i.e.*, with formic acid and formate dehydrogenase,[4] or electrochemically mediated regeneration of NADPH.[6] Reduced nicotinamide also plays an important role as an electron carrier in the photosynthetic apparatus leading to the fixation of CO_2 to carbohydrates.[7] Thus, it might be of special interest to develop photochemical systems for the regeneration of NAD(P)H as a means of solar energy conversion and storage. The photosensitized production of NADH has been mediated by $Rh(bpy)_3^{3+}$ with a homogeneous photosensitizer[8] or TiO_2 semiconductor particles[9] as the light absorbants. We have recently reported[1] on the cyclic photosensitized enzyme-catalysed production of NADPH using ruthenium(II)trisbipyridine, $Ru(bpy)_3^{2+}$, as sensitizer, ammonium ethylenediaminetetra-acetic acid, $(NH_4)_3EDTA$, as electron donor, *NN'*-dimethyl-4,4'-bipyridinium, MV^{2+}, as mediating electron carrier, and ferredoxin-$NADP^+$-reductase (E.C.1.18.1.2) as enzyme catalyst. With this system we successfully reduced butan-2-one to the corresponding alcohol by coupling the photoproduced NADPH with a secondary alcohol dehydrogenase enzyme catalyst. In this paper we present a comprehensive study of the photosensitized regeneration of 1,4-dihydronicotinamide adenine dinucleotide phosphate, NADPH, using $Ru(bpy)_3^{2+}$ as photosensitizer, and of 1,4-dihydronicotinamide adenine dinucleotide, NADH, with zinc(II)meso-tetramethylpyridinium-porphyrin, $ZnTMPyP^{4+}$, as sensitizer. We describe the application of the NAD(P)H regeneration systems in a variety of synthetic routes.

Experimental

Absorption spectra were recorded with a Uvikon-820 (Kontron) spectrophotometer equipped with a ψ-80 (Kontron) computer. Gas chromatographic analysis was performed with a Tracor-540 gas chromatograph. Liquid chromatography analyses were performed with a Perkin-Elmer series 4 HPLC apparatus, or in an LKB 4400 amino acid analyser. The optical purity was determined by a Perkin-Elmer 141 polarimeter.

Illumination was performed at room temperature in a glass cuvette (4 ml) equipped with a small magnetic stirrer, a valve, and a serum stopper with a 1000 W halogen–quartz lamp. Light was filtered through a 400 nm cutoff filter (Kodak 2C), photon flux 7.1×10^{-3} einstein l^{-1} min^{-1}.

The production of NAD(P)H and $MV^{+\cdot}$ was followed spectroscopically at λ 340 (ε 6.2×10^3 $l\,cm^{-1}$ mol^{-1}) and 602 nm (ε 1.25×10^4), respectively. The progress of the reactions under continuous illumination was determined by chromatographic means specified for each product.

All chemicals and biochemicals were obtained from Sigma or Aldrich. Rutheniumtrisbipyridine dichloride,[10] $Ru(bpy)_3Cl_2$, zinc(II)meso-tetramethylpyridiniumprophyrin iodide,[11] $ZnTMPyPI_4$, and sodium acetoacetate were prepared in our laboratory, according to published procedures.

A solution of sodium acetoacetate was prepared according to the literature[12] and evaporated to dryness. The product was microanalysed and checked to yield NAD^+ by adding NADH and β-hydroxybutyrate dehydrogenase.

Samples (3 ml) consisting of all the components were transferred to the glass cuvette (4 ml) and were deaerated by repeated evacuation followed by oxygen free argon flushings.

Butan-2-ol.—The system was composed of an aqueous 0.1M-tris buffer soluion (pH 7.8) that included $Ru(bpy)_3^{2+}$ (7.5×10^{-5}M), ammonium ethylenediaminetetra-acetic acid [$(NH_4)_3$-EDTA, 2×10^{-2}M initial concentration], $NADP^+$ (1×10^{-3}M), methyl viologen (MV^{2+}, 1×10^{-3}M), 2-mercaptoethanol (1×10^{-3}M), and the substrate butan-2-one (0.15M). The sample (3 ml) also contained ferredoxin-$NADP^+$-reductase (FDR; E.C. 1.18.1.2; 0.5 units) and alcohol dehydrogenase (ALDH; E.C. 1.1.1.2; 10 units from *T. Brockii*[13]). The deaerated system was illuminated, and the rate of butan-2-ol formation was followed by gas chromatography using a Porapak T column (2 m, oven temperature 175 °C, N_2 flow rate 50 ml min^{-1}). Optical purity was determined at λ 589 nm.

Glutamic Acid.—The system was composed of an aqueous 0.1M-Tris buffer solution (3.75 ml; pH 7.9), that included

$Ru(bpy)_3^{2+}$ (1×10^{-4}M), 2-mercaptoethanol (2×10^{-2}M, initial concentration), $NADP^+$ (8.8×10^{-4}M), MV^{2+} (1.76×10^{-3}M), NH_4^+ (0.1M), and the substrate α-oxoglutarate (0.1M). This system included also FDR (E.C. 1.18.1.2; 0.5 units) and glutamic acid dehydrogenase (GluDH; E.C. 1.4.1.3; 22 units). The deaerated system was illuminated ($\lambda > 400$ nm). The rate of glutamic acid formation was followed by an amino acid analyser. Each sample (100 μl) was treated before analysis with 5-sulphosalicyclic acid as described elsewhere,[14] in order to precipitate the enzymes. The optical purity of the product solution was determined at λ 546 nm. The product solution was then passed through a Dowex 50W × 8 cation-exchange resin that removed glutamic acid. The optical activity of the resulting solution was determined to subtract any background activity.

Lactic Acid.—A solution containing Tris buffer (0.1M; 3 ml; pH 7.5), was composed of $ZnTMPyP^{4+}$ (1.3×10^{-5}M), 2-mercaptoethanol (1×10^{-2}M, initial concentration), NAD^+ (1×10^{-3}M), MV^{2+} (4×10^{-3}M), and the substrate sodium pyruvate (0.1M). To this sample were introduced lipoamide dehydrogenase (LipDH; E.C. 1.6.4.3; 20 units) and lactic dehydrogenase (LDH; E.C. 1.1.1.27; 250 units). The deaerated solution was illuminated and product formation was followed at time intervals of illumination. The rate of lactate formation was followed by gas chromatography using modification of the reported procedure:[15] samples (10 μl) were dissolved in oxalic acid (90 μl; 0.1M) and were injected into a 1 m coil glass column, Tenax G.C. 60/80 (Alltech). The optical purity was determined at λ 546 nm.

Alanine.—The system was composed of an aqueous 0.1M-Tris buffer solution (3 ml; pH 7.9) that included $ZnTMPyP^{4+}$ (1×10^{-5}M), 2-mercaptoethanol (1×10^{-2}M initial concentration), NAD^+ (1.4×10^{-3}M), MV^{2+} (4×10^{-3}M), NH_4^+ (0.1M), and the substrate sodium pyruvate (0.1M). To this sample were added lipoamide dehydrogenase (LipDH; E.C. 1.6.4.3; 100 units) and alanine dehydrogenase (AlaDH; E.C. 1.4.1.1; 0.25 units). The deaerated system was illuminated and product formation was followed at time intervals of illumination. The rate of alanine formation was followed by liquid chromatography using a procedure developed recently by Grushka and Levine.[16] Each sample to be determined was dissolved in the h.p.l.c. eluent (10 times its volume) and filtered (milipore 0.22μ) before it was injected. The optical purity was determined at λ 546 nm.

β-Hydroxybutyric Acid.—To a solution of Tris buffer (3.7 ml; 0.1M; pH 7) were introduced the following components: $ZnTMPyP^{4+}$ (9.7×10^{-6}M), 2-mercaptoethanol (1.88×10^{-2}M, initial concentration), NAD^+ (9.4×10^{-4}M), MV^{2+} (3.9×10^{-3}M), and the substrate, sodium acetoacetate (5×10^{-2}M). To this solution were added lipoamide dehydrogenase (LipDH; E.C. 1.6.4.3; 100 units) and β-hydroxybutyrate dehydrogenase (β-HBuDH; E.C. 1.1.1.30; 2 units). The rate of the product *e.g.* β-hydroxybutyrate formation was followed by gas chromatography (Tenax G.C. 60/80) using the same procedure as for lactic acid. The only difference was the oven temperature which was set to 165 °C. The samples were also diluted (1:10) in oxalic acid (0.1M) before injection. The optical purity was determined as described previously.[17]

Results and Discussion

Photosensitized NAD(P)H Formation.—*NN'*-Dimethyl-4,4'-bipyridinium radical cation, $MV^{+\bullet}$ (methyl viologen radical), mediates the enzyme-catalysed reduction of $NAD(P)^+$ to NAD(P)H. Lipoamide dehydrogenase (E.C. 1.6.4.3) and ferredoxin-$NADP^+$-reductase (E.C. 1.18.1.2) catalyse the production of NADH and NADPH respectively.[18,19] The photosensitized production of $MV^{+\bullet}$ has been extensively studied in the past few years as a means of solar energy conversion and storage.[20,21] In these systems $Ru(bpy)_3^{2+}$ or zinc-porphyrin act as photosensitizers. Thus, it seems reasonable to suggest that the coupling of the photosensitized reactions with the enzyme-catalysed processes might offer a novel route for the development of NAD(P)H regeneration cycles.

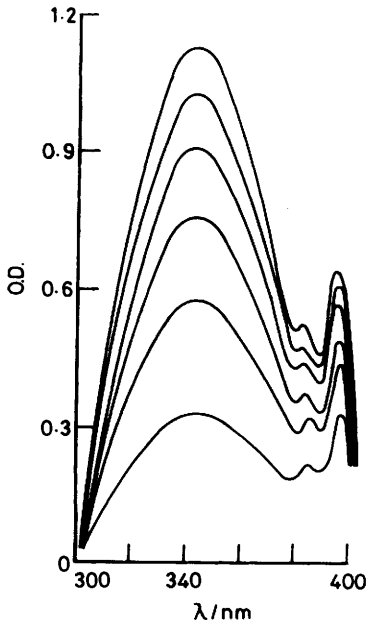


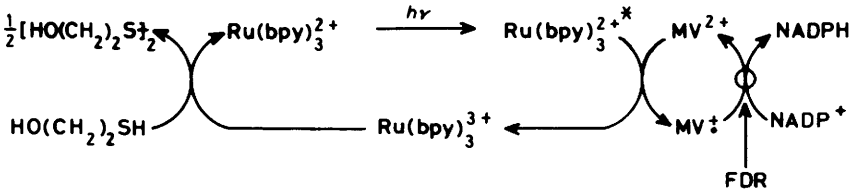
Figure 1. NADPH formation at time intervals of 20 s of illumination: 0.1M-Tris buffer at pH 7.8 containing $[Ru(bpy)_3^{2+}]$ 6.8×10^{-5}M, $[MV^{2+}]$ 1×10^{-3}M, $[NADP^+]$ 1×10^{-3}M, [2-mercaptoethanol] 2×10^{-2}M, and 0.25 units of FDR

Illumination of an aqueous solution (pH 7.8) that includes the sensitizer, $Ru(bpy)_3^{2+}$, *NN'*-dimethyl-4,4'-bipyridinium, MV^{2+}, $NADP^+$, enzyme ferredoxin-$NADP^+$-reductase, $(NH_4)_3EDTA$, or 2-mercaptoethanol as electron donor results in the formation of NADPH (Figure 1). The rate of NADPH formation was followed at λ 340 nm and corresponds to a quantum yield of φ 1.9%. Exclusion of the enzyme ferredoxin-$NADP^+$-reductase eliminates the formation of NADPH. Furthermore, it can be seen from Figure 1 that in the presence of the enzyme ferredoxin-$NADP^+$-reductase, $MV^{+\bullet}$ is in equilibrium with NADPH [equation (1)]. The equilibrium

$$2MV^{+\bullet} + NADP^+ + H^+ \rightleftharpoons 2MV^{2+} + NADPH \quad (1)$$

constant of this process has been estimated to be $K = (8 \pm 3) \times 10^{10}$ l mol^{-1} at 26 °C and pH 7.8. These results imply that $MV^{+\bullet}$ acts as an electron carrier that mediates the enzyme-catalysed production of NADPH (Scheme 1). In this cycle the excited sensitizer is quenched by MV^{2+} *via* electron transfer. Charge separation of the electron-transfer products results in the formation of $MV^{+\bullet}$ and $Ru(bpy)_3^{3+}$. Subsequent oxidation of $(NH_4)_3EDTA$ by the oxidized photoproduct regenerates the sensitizer and the cycle is completed. The reduced photoproduct, $MV^{+\bullet}$, mediates the reduction of $NADP^+$ in the presence of ferredoxin-$NADP^+$-reductase as enzyme catalyst.

The photosensitized production of NADH has been similarly examined in a system including $Ru(bpy)_3^{2+}$ as sensitizer, MV^{2+} as electron carrier, $(NH_4)_3EDTA$ as electron donor, the enzyme lipoamide dehydrogenase (E.C. 1.6.4.3), and NAD^+. Illumination of this system results in only low yields of NADH.



Scheme 1. Photosensitized production of NADPH

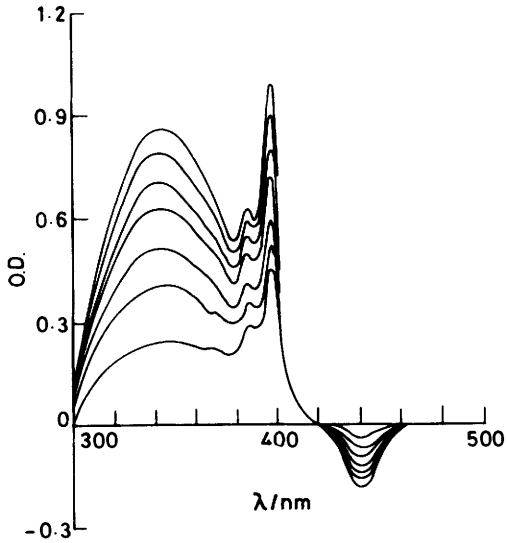


Figure 2. NADH formation at time intervals of 20 s of illumination (each spectrum was recorded after equilibrating the system for 1 min in the dark): 0.1M-Tris buffer at pH 8 containing [$ZnTMPyP^{4+}$] 6.5 × 10^{-6}M, [MV^{2+}] 4 × 10^{-3}M, [NAD^+] 1 × 10^{-3}M, [2-mercaptoethanol] 2 × 10^{-2}M, and 100 units of LipDH

The low yields of NADH are attributed to the electron-donation properties of NADH. Illumination of a system composed of the sensitizer, $Ru(bpy)_3^{2+}$, MV^{2+}, and NADH results in the photosensitized formation of $MV^{+\cdot}$. Since NADH does not quench the excited $^*Ru(bpy)_3^{2+}$, the formation of $MV^{+\cdot}$ is attributed to the oxidation of $Ru(bpy)_3^{3+}$, the formation of $MV^{+\cdot}$ is attributed to the reduction of $Ru(bpy)_3^{3+}$ by NADH.* Consequently, NADH is consumed and its subsequent utilization in the reduction of substrate is prevented. Substitution of the photosensitizer $Ru(bpy)_3^{2+}$ with zinc(II)meso-tetramethylpyridiniumporphyrin, $ZnTMPyP^{4+}$, results in the effective formation of NADH. The rate of formation of NADH upon illumination of an aqueous system which includes $ZnTMPyP^{4+}$ results in the effective formation of NADH. The rate of fomation of NADH upon illumination of an aqueous system which includes $ZnTMPyP^{4+}$ as photosensitizer, MV^{2+} as electron acceptor, 2-mercaptoethanol as electron donor, NAD^+, and the enzyme lipoamide dehydrogenase (E.C. 1.6.4.3) is shown in Figure 2. It can be seen that NADH is formed at time intervals of illumination (λ 340 nm). Also in equilibrium with NADH is a small amount of $MV^{+\cdot}$ (λ 395 nm). We should however note that the photosensitizer $ZnTMPyP^{4+}$ undergoes degradation upon illumination as indicated by the negative absorption spectra at λ 438 nm. With 2-mercaptoethanol as electron donor 14% of the original photosensitizer is photodegraded upon formation of 1.15 × 10^{-4}M-NADH. Previous studies have indicated[22] that zincporphyrin undergoes irreversible photodegradation in the presence of electron donors such as EDTA. Indeed, we find that, with $(NH_4)_3EDTA$ or triethanolamine as electron donors in the system, NADH is formed although the degradation of the sensitizer, $ZnTMPyP^{4+}$, is accelerated. We find that 2-mercaptoethanol exhibits the preferred behaviour in acting as an effective electron donor for NADH production and minimizing the degradative pathway.

Exclusion of the enzyme lipoamide dehydrogenase or MV^{2+} eliminates the production of NADH. Addition of the enzyme to an aqueous solution that includes the photogenerated $MV^{+\cdot}$ and NAD^+ results in the disappearance of $MV^{+\cdot}$ and the concomitant formation of NADH. These results imply that $MV^{+\cdot}$ functions as an electron carrier that mediates the product of NADH in the presence of the enzyme lipoamide dehydrogenase,[19] [equation (2) and Scheme 2]. The quenching of the excited sensitizer $ZnTMPyP^{4+}$ forms $MV^{+\cdot}$ and the oxidized sensitizer. Subsequent oxidation of 2-mercaptoethanol regenerates the light-active substance and $MV^{+\cdot}$ mediates the production of NADH. From the amount of $MV^{+\cdot}$ that is in equilibrium with NADH (Figure 2) the equilibrium constant of the process outlined in equation (2) is estimated to be $K = (6 \pm 2) \times 10^{11}$ l mol^{-1}.

$$2MV^{+\cdot} + NAD^+ + H^+ \rightleftharpoons 2MV^{2+} + NADH \quad (2)$$

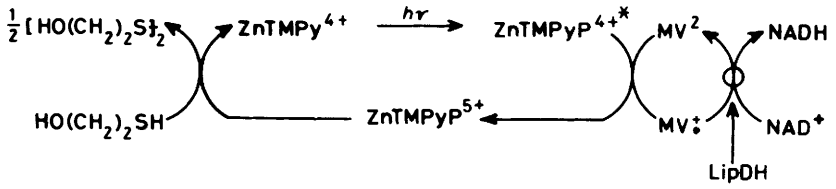
There is however a significant difference between the photoinduced NADPH and NADH regeneration cycles. The quantum yields of $MV^{+\cdot}$ in the presence of $(NH_4)_3EDTA$ and of 2-mercaptoethanol are equal, φ 3.8%. In the presence of ferredoxin-$NADP^+$-reductase (0.25 units), NADPH is immediately formed upon illumination, φ 1.9%. Thus, the quantum yield for NADPH formation is half the value of that for $MV^{+\cdot}$, consistant with equation (1). In the NADH regeneration cycle, $MV^{+\cdot}$ accumulates upon illumination and the reduction of NAD^+ to NADH in the presence of lipoamide dehydrogenase (100 units) is relatively inefficient. Therefore, the accumulation of NADH as a function of time intervals of illumination (Figure 2) was measured after the system was allowed to equilibrate for 1 min in the dark according to equation (2). Thus, it appears that under the experimental conditions employed the NADH regeneration cycle is inefficient as compared with the NADPH cycle, despite the higher amount of enzyme units for NADH. We thus believe that under these conditions the rate-limiting reaction for NADPH production is the photochemical electron-transfer process, while for NADH the enzyme-catalysed reduction of NAD^+ by $MV^{+\cdot}$ is the rate-determining reaction.

Utilization of Photosensitized Regeneration Systems in Synthesis.—Many enzymatic reduction processes are dependent on the NAD(P)H cofactors.[2] Thus coupling of the photosensitized NAD(P)H regeneration cycles with secondary enzyme-catalysed processes is expected to allow various synthetic applications. The photosensitized NADPH regeneration system

* The formation of $MV^{+\cdot}$ in $Ru(bpy)_3^{2+}$ as sensitizer and NADH as electron donor is summarized in equations (i) and (ii).

$$^*Ru(bpy)_3^{2+} + MV^{2+} \longrightarrow Ru(bpy)_3^{3+} + MV^{+\cdot} \quad (i)$$

$$Ru(bpy)_3^{3+} + NADH \longrightarrow Ru(bpy)_3^{2+} + NAD^{\cdot} + H^+ \quad (ii)$$



Scheme 2. Photosensitized production of NADH

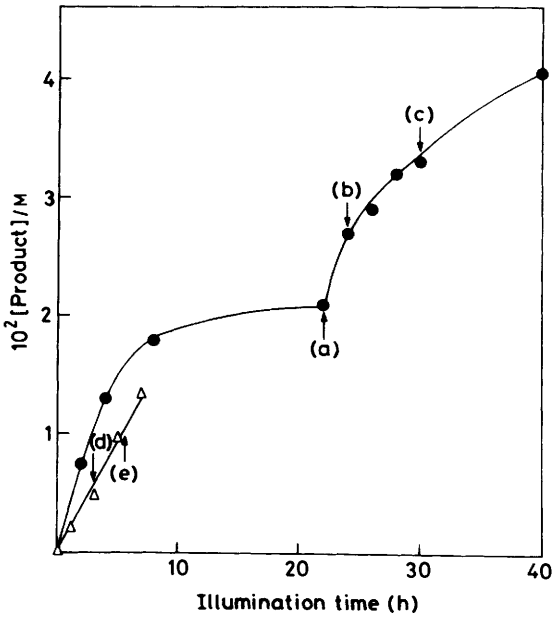
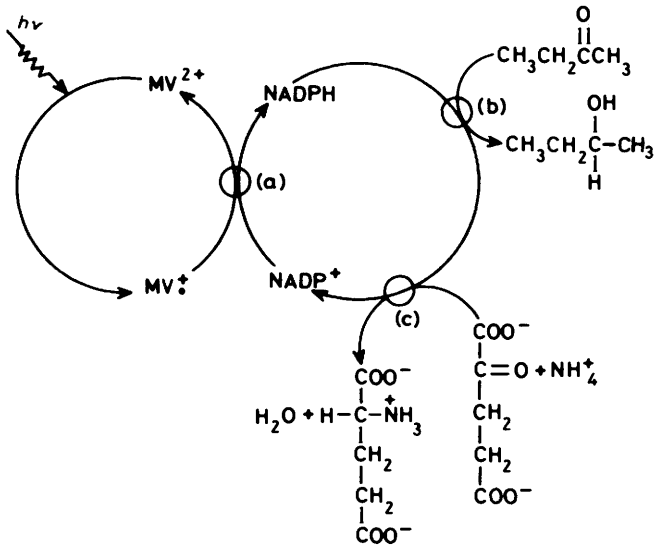


Figure 3. Rate of butan-2-ol (●) and of glutamic acid (△) formation as a function of illumination time: (a) addition of $(NH_4)_3EDTA$, 2×10^{-2}M; (b), (c) addition of $(NH_4)_3EDTA$, 1.7×10^{-2}M; (d) addition of 2-mercaptoethanol, 1.2×10^{-2}M; (e) addition of 2-mercaptoethanol, 1.9×10^{-2}M



Scheme 3. Enzyme-catalysed reactions coupled to the photoinduced NADPH regeneration system: (a) FDR; (b) AlaDH; (c) GluDH

that includes the sensitizer $Ru(bpy)_3^{2+}$, MV^{2+} as electron acceptor, an electron donor, $NADP^+$, and the enzyme ferredoxin-$NADP^+$-reductase was coupled with NADPH-dependent enzymes. The systems that have been examined are the reduction of butan-2-one to butan-2-ol catalysed by the enzyme alcohol dehydrogenase (E.C. 1.1.1.2 from *T. Brockii*) and the reductive amination of α-oxoglutaric acid to glutamic acid catalysed by glutamic dehydrogenase (E.C. 1.4.1.3). The NADPH regeneration system includes $(NH_4)_3EDTA$ as electron donor for the reduction of butan-2-one. However, since glutamic dehydrogenase is deactivated by EDTA,[23] we have used 2-mercaptoethanol as electron donor for the photosensitized regeneration of NADPH in the reductive amination of α-oxoglutaric acid. Illumination of these systems results in the formation of butan-2-ol and glutamic acid (Figure 3). After 40 h of illumination 27% of butan-2-ol is formed and after 7 h of illumination 13% glutamic acid is formed. With butan-2-one as substrate it can be seen that after 10 h of illumination the rate of butan-2-ol production levels off. This is a result of the consumption of the electron donor $(NH_4)_3EDTA$. At low concentrations of the electron donor the back-electron-transfer reaction of the intermediate photoproducts $Ru(bpy)_3^{3+}$ and $MV^{+\cdot}$ predominates and the subsequent production of NADPH is eliminated. Addition of $(NH_4)_3EDTA$ to the system restores the activity of the system towards reduction of butan-2-one and by constant addition of the electron donor the initial high rate of butan-2-ol formation is maintained (Figure 3).

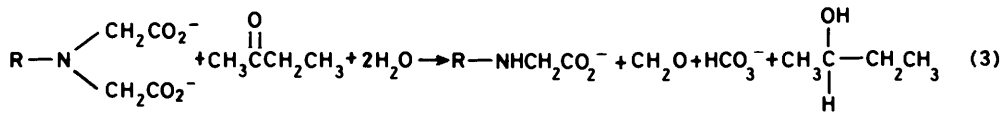
In such artificial systems that include enzymes as catalysts several aspects must be considered. (a) The first is the stability of the enzymes in the artificial environment, namely the turnover of the enzymes. (b) Since enzymes are acting as catalysts, prochiral substrates are expected to yield chiroselective products. The enantiomeric excess (% e.e.) is thus of special interest. The Table summarizes the estimated turnover numbers for the ingredients in the systems that produce (+)-butan-2-ol and L-glutamic acid. These turnovers were estimated after 40 h of illumination for butan-2-ol and 7 h of illumination for glutamic acid. It should be noted that no loss in the activity of the systems could be detected after these illumination periods. Thus, it is evident that the enzymes and cofactor, as well as the artificial ingredients, are perfectly stable in multi-component systems and are effectively recycled in the photoinduced process. The optical purity of the products formed in these enzyme-catalysed reactions is also summarized in the Table. It can be seen that the % e.e. of butan-2-ol and of glutamic acid is 100 ± 10 and 99 ± 7%. These high optical purities emphasize that the artificial environments and the photochemical reactions do not affect the chiroselectivity of the enzymatic processes.

Exclusion of the enzymes alcohol dehydrogenase or glutamic acid dehydrogenase from the systems results in the accumulation of NADPH and prevents the formation of products. Addition of the enzymes to the systems results in the consumption of NADPH and formation of products. These results imply that NADPH mediates the reduction of butan-2-one and α-oxaglutaric acid in the presence of the enzyme catalysts, and that the primary photoinduced NADPH regeneration cycle can be coupled to secondary enzyme-catalysed reactions (Scheme 3).

Table. Turnover numbers (TN) of the components in the different enzyme-catalysed reactions

	$Ru(bpy)_3^{2+}$	$ZnTMPyP^{4+}$	MV^{2+}	$NAD(P)^+$	Enzymes		% Conversion	% Optical purity	Final product concentration (M)	Initial substrate concentration (M)
Butan-2-ol	530		40	40	FDR[a] 2.4×10^4	ALDH[c] 6×10^3	27	100 ± 10	4.05×10^{-2}	0.15
Glutamic acid	225		15	15	FDR 9.75×10^3	GluDH[d] 1.26×10^5	13	99 ± 7	1.3×10^{-2}	0.1
Lactic acid		4 500	13.5	27	LipDH[b] 5.67×10^4	LDH[e] 4.15×10^4	27	94 ± 7	2.7×10^{-2}	0.1
Alanine		7 900	19	27	LipDH 1.7×10^4	AlaDH[f] 3.2×10^6	38	78 ± 7	3.8×10^{-2}	0.1
β-Hydroxybutyric acid		1 900	2.4	10	LipDH 5.2×10^3	β-HBuDH[g] 8.3 × 10	21	105 ± 10	9.4×10^{-3}	4.52×10^{-2}

[a] F.W. ≃ 40 000 (M. Shin, *Methods Enzymol*, 1971, **23**, 441). [b] F.W. ≃ 70 000 (F. B. Straub, *Biochem. J.*, 1939, **33**. 787). [c] F.W. ≃ 40 000.[11] [d] F.W. ≃ 2 200 000 (H. Sund and W. Burchard, *Eur. J. Biochem.*, 1968, **6**, 202). [e] F.W. ≃ 140 000 (R. Jaenicke and S. Knof, *Eur. J. Biochem.*, 1968, **4**, 157). [f] F.W. ≃ 228 000 (A. Yoshida and G. Freese, *Biochim. Biophys. Acta*, 1964, **92**, 33). [g] F.W. ≃ 850 000 (H. U. Bergmeyer, *Biochem. J.*, 1967, **102**, 423).



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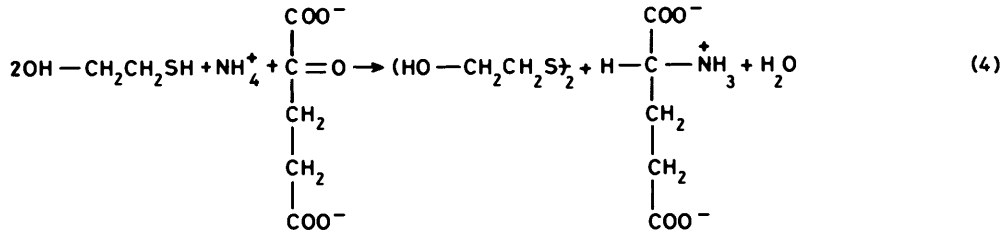

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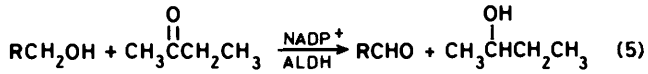


(5)

The overall reactions accomplished in the NADPH-mediated reduction processes correspond to the reduction of butan-2-one by $(NH_4)_3EDTA$ [equation (3)] and to the reductive amination of α-oxoglutarate by 2-mercaptoethanol [equation (4)]. These processes are estimated to be endoergic by *ca.* 33 and 24 kcal mol^{-1}, respectively.

In principle, similar reduction reactions of the substrates could be accomplished by the regeneration of NADPH with reducing agents.[4,5] For example, the reduction of butan-2-one by methanol has been reported in the presence of alcohol dehydrogenase and $NADP^+$ [equation (5)].[11] Nevertheless, this system will reach equilibrium due to reversibility of the system. The present photochemical system is clearly advantageous since it is capable of driving product formation towards completion *via* continuous conversion of light energy into chemical potential, which is stored in NADPH and subsequently in the products.

The photosensitized NADH regeneration cycle, using Zn-$TMPyP^{4+}$ as sensitizer, was similarly coupled to reduction processes catalysed by NADH-dependent enzymes. We have examined the reduction of two oxo-acids, *i.e.* reduction of pyruvic acid to lactic acid catalysed by lactate dehydrogenase (E.C. 1.1.1.27), and reduction of acetoacetic acid with β-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30) to β-hydroxybutyric acid. Similarly, the reductive amination of pyruvic acid in the presence of NH_4^+ to alanine has been studied with alanine dehydrogenase (E.C. 1.4.1.1). Illumination of an aqueous solution of zinc(II)meso-tetramethylpyridiniumporphyrin, MV^{2+}, NAD^+, and 2-mercaptoethanol as electron donor, with one of the substrates pyruvate or acetoacetate and the corresponding enzymes, results in the formation of L-lactic acid, D-β-hydroxybutyric acid, and L-alanine, respectively. The rate of production of these products as a function of illumination time is displayed in Figure 4. It can be seen that the rate of product formation levels off as illumination proceeds. These results are due to two complementary factors: (a) consumption of the electron donor, and (b) slow degradation of the light-active species, the photosensitizer. At low concentrations of the electron donor, the back-electron-transfer reaction of the primary photoproducts, the oxidized sensitizer, and $MV^{+\bullet}$, competes effectively with the sacrificial route of donor oxidation by the oxidized sensitizer. Consequently, the subsequent formation of NADH and products is prevented. Recovery of the initial concentration of the electron donor regenerates the production of NADH and products. The second difficulty encountered in these systems is the slow, but significant, degradation of the sensitizer. This problem has been emphasized in the previous section and will be further discussed later.

The turnover numbers of the artificial and natural ingredients in the different systems utilizing NADH are summarized in the Table. It can be seen that the enzymes and cofactor are effectively recycled in the systems. For example, when 38% conversion of pyruvic acid into alanine is accomplished the

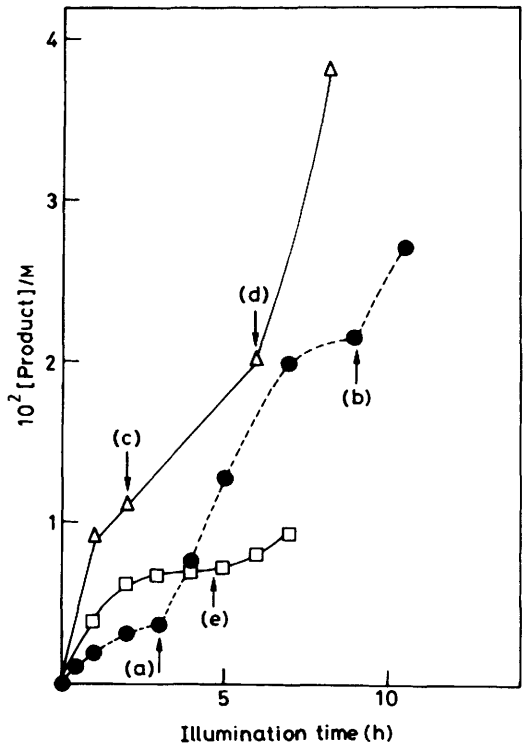


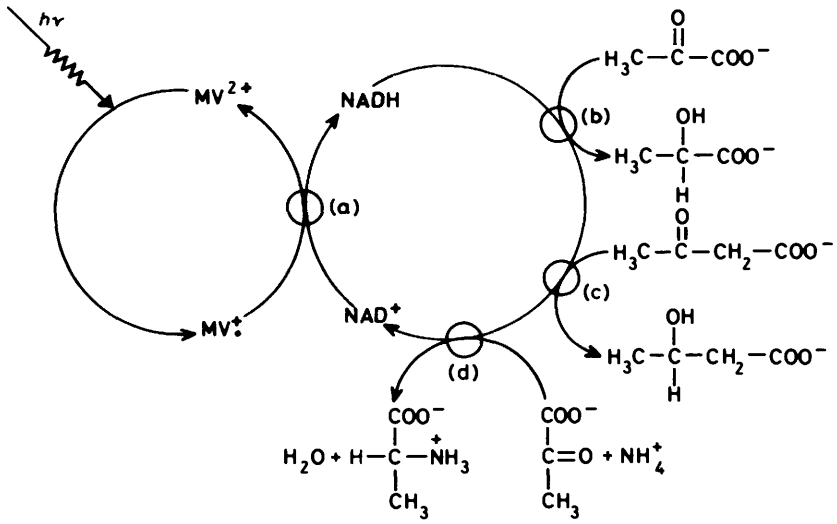
Figure 4. Rate of lactic acid (●), alanine (△), and β-hydroxybutyric acid (□) as a function of illumination time: additions of 2-mercaptoethanol, (a) and (b) 3.3×10^{-2}M each; (c) 4×10^{-2}M; (d) 2.3×10^{-2}M; (e) 1×10^{-2}M

substitute the present sensitizer by other light-active substances in order to develop long term stable NADH regeneration cycles.

Nevertheless it should be noted that the stability of the photosensitizer is increased in the presence of the secondary enzymatic cycle that utilizes the photogenerated NADH in the reduction of the various substrates. In the previous section it was shown (Figure 2) that the photosensitized production of NADH is accompanied by the degradation of the photosensitizer, $ZnTMPyP^{4+}$. From the depletion of the absorbance of $ZnTMPyP^{4+}$ we estimate that 14% of the sensitizer is degraded when 1.15×10^{-4}M-NADH are formed. When the secondary enzymatic cycle is coupled to the NADH regeneration cycle this degradation process is retarded. For example, with pyruvic acid as substrate and lactate dehydrogenase 2×10^{-2}M-lactic acid is formed (corresponding to 2×10^{-2}M-NADH) and only 17% of the sensitizer is degraded. These results demonstrate that the sensitizer is substantially stabilized in the presence of the secondary enzymatic system and that the accumulation of $MV^{+\cdot}$ and NADH affects the degradation of the sensitizer.

Attention must be given to the optical activity of the products formed by the NADH regeneration system. The Table summarizes the enantiomeric excess of the products formed upon illumination of the different systems. It can be seen that high chiroselectivity in the products is obtained under the photochemical conditions and the conjugation of artificial and enzymatic components.

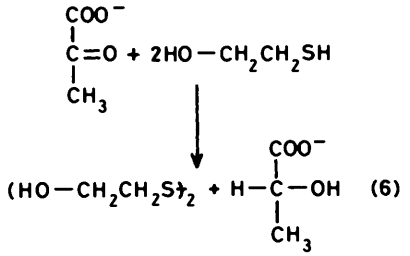
Exclusion of the NADH-dependent enzymes from the systems eliminates the reduction of pyruvic acid and acetoacetic acid and the reductive amination of pyruvic acid. We thus conclude that introduction of a variety of NADH-dependent enzymes (Scheme 4) allows the utilization of the photosensitized NADH regeneration cycles in synthetic routes.



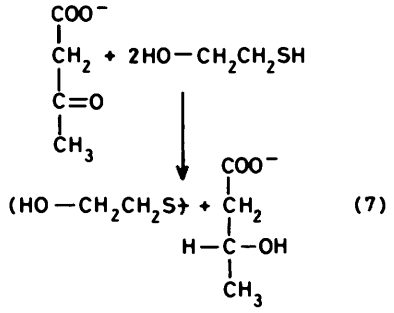
Scheme 4. Enzyme-catalysed reactions coupled to the photosensitized NADH regeneration cycle. (a) LipDH; (b) LDH, (c) β-H-BuDH; (d) AlaDH

enzymes lipoamide dehydrogenase and alanine dehydrogenase are recycled 1.7×10^4 and 3.2×10^6 times, without affecting their activities. The major difficulty is encountered with the photosensitizer, $ZnTMPyP^{4+}$. When 20% of pyruvic acid is converted into lactic acid, corresponding to a turnover of 3 300 for the sensitizer, 17% of the light-active compound is degraded. Thus, to accomplish high conversion yields of the products, addition of the sensitizer is required. It seems desirable to

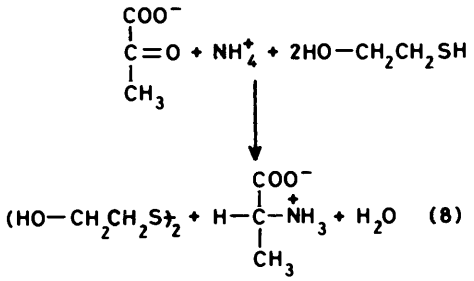
The net reactions that are mediated by the photosensitized electron-transfer process and NADH correspond to the reduction of pyruvate or acetoacetate by 2-mercaptoethanol [equations (6) and (7)] or to the reductive amination of pyruvate to L-alanine by 2-mercaptoethanol [equation (8)]. The energetic balance of these processes has been estimated to be ΔG 7 kcal mol^{-1} for lactate formation, ΔG 15 kcal mol^{-1} for β-hydroxybutyrate, and ΔG *ca.* 0 for alanine production. Thus,



(6)



(7)



(8)

the application of light energy as a means of driving endoergic processes is evident in the synthesis of L-lactate and of β-hydroxybutyrate.

Conclusions.—We have developed photochemically induced NADH and NADPH regeneration systems. For NADH production, zinc(II)meso-tetramethylpyridiniumporphyrin is used as sensitizer. This sensitizer is photochemically unstable and undergoes photoinduced degradation. Thus for the long-term activity of NADH regeneration systems the substitution of this photosensitizer is required. For NADPH formation, ruthenium(II)trisbipyridine is used as photosensitizer. This compound is stable under the photochemical conditions and does not undergo any noticeable degradation. Also, in contrast to the effective NADPH formation by $MV^{+\bullet}$ and the enzyme ferredoxin-$NADP^+$-reductase, a similar process for NADH is relatively slow in the presence of the enzyme lipoamide dehydrogenase. It seems desirable to substitute MV^{2+} with another electron mediator for enhancing the production of NADH.

As various processes in nature are catalysed by NAD(P)H-dependent enzymes, many biotechnological applications of photoinduced NAD(P)H regeneration cycles might be envisaged, *i.e.* chiroselective or stereoselective synthesis. Of particular interest is the application of photosensitized NAD(P)H regeneration cycles in the stepwise fixation of CO_2 to methanol. These processes will involve the introduction of several NAD(P)H-dependent enzymes such as formate dehydrogenase, aldehyde dehydrogenase, and alcohol dehydrogenase. These different aspects are currently being examined in our laboratory.

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