CHEMISTRY *and* BIOCHEMISTRY *of* FLAVOENZYMES

Volume III

Franz Müller

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Chemistry and Biochemistry of Flavoenzymes

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Editor

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PREFACE

Flavin was discovered about a century ago. Since its discovery, the field of flavin and flavoproteins has developed into a mature science. Although a few flavoproteins had already been discovered during the first three decades of the 20th century, a profound understanding of the enzymic reaction mechanisms of flavoproteins became possible only after full development of the chemical and physical properties of free flavin. This development was initiated about 60 years ago and was mainly concerned with the chemical synthesis of the flavin molecule. Research in the field of flavins and flavoproteins was discontinued during the Second World War, but revived in the 1950s. The postwar period brought increased progress in the chemistry of free flavin, which knowledge was instrumental in understanding the biochemical properties of flavoproteins.

Flavoproteins are involved in a variety of biological reactions including electron transfer, oxidation, dehydrogenation, and monooxygenation reactions. Many flavoproteins contain flavin as the sole prosthetic group, but the reaction mechanisms can still be complex. Other flavoproteins are composed of flavin and heme and non-heme iron, molybdenum and other metal ions, or pteridin. Although they represent a minority, flavoproteins resulting from covalent bondings of the prosthetic group to amino acid residues of proteins have enhanced the variety of flavoproteins.

Present knowledge in the field discussed in this work would not be possible without advancements in biophysical equipment. New techniques have contributed a great deal to the elucidation of primary, secondary, tertiary, and quaternary structures of flavoproteins. In recent years the three-dimensional structures of a few flavoproteins have become available, and it can be expected that this research will continue to advance. These data will provide the basis for new research on flavoproteins — namely, cloning of genes, encoding for flavoproteins, followed by site-directed mutagenesis of flavoproteins. This research is presently in its infancy, but is expected to develop and provide even deeper insight into the enzymic mechanisms of flavoproteins and the biochemical principles governing the catalytic action of particular flavoenzymes.

Given the wealth of data available on flavins and flavoproteins, it seems appropriate to report on the progress of a century of research in the field. In the present work an attempt has been made to summarize this knowledge as comprehensively as possible. Despite limitations of space and unavailability of some material, this and succeeding volumes of the *Chemistry and Biochemistry of Flavoenzymes* should be a valuable reference for researchers in the field and those entering it, as well as a source for lecturers in biochemistry, biophysics, chemistry, pharmacology, toxicology, and medicine.

In conclusion I wish to thank all contributors for their cooperation and their efforts to meet our deadlines, and I would also like to express personal appreciation to my wife, Rita, and our daughters, Sandra and Kirsten, for their patience over many weekends in the development of this series.

Franz Müller

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Dr. Müller obtained his training at the University of Basle, and received his M.S. degree in 1961 and Ph.D. degree in 1964 from the Departments of Organic and Inorganic Chemistry. After doing postdoctoral work at the Medical Nobel Institute, Department of Biochemistry, Stockholm, Sweden, he was first a Research Associate in the Department of Biological Chemistry at the University of Michigan in Ann Arbor, and later Instructor, and a recipient of a Career Research Development Award from the National Institutes of Health. In 1971 he was appointed an Associate Professor of Biochemistry at the Agricultural University, Wageningen, The Netherlands, and Research Professor in 1977, where he served as Chairman of the Department of Biochemistry and chaired the university's Science Committee and the Committee for Biotechnology. In 1987 he assumed his present position.

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Chapter 1

PYRIDOXINE-5-P OXIDASE

Francis Kwok and Jorge E. Churchich

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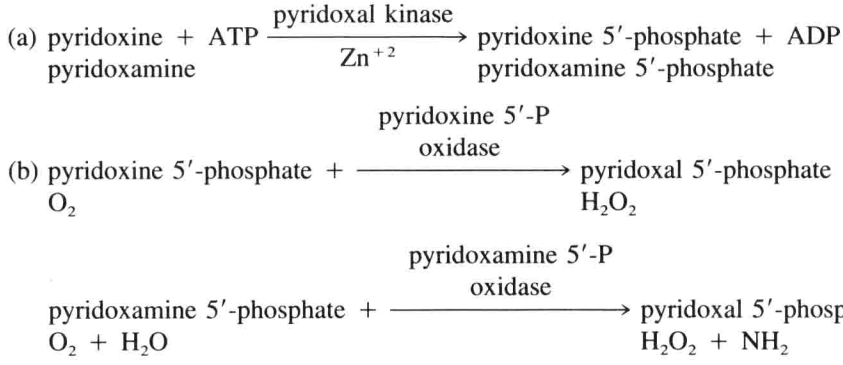
I. INTRODUCTION

Pyridoxal 5′-phosphate, the metabolically active form of vitamin B_6, is the coenzyme required by numerous enzymes involved in transamination, and racemization reactions. Two reaction steps are included in the conversion of pyridoxine and pyridoxamine to pyridoxal 5′-phosphate: (1) phosphorylation catalyzed by pyridoxal kinase and (2) oxidation of the phosphorylated vitamins catalyzed by the FMN-dependent pyridoxine (pyridoxamine) 5′-phosphate oxidase. The reactions are shown as in the following:



Pyridoxine (pyridoxamine) 5′-P oxidase (EC1.4.3.5) was first found in rabbit liver by Pogell[1] and later studied by many other laboratories including the laboratory of Wada and Snell.[2] The localization of this enzyme in mammals covers a wide range of tissues including liver, kidney, and brain with high activities; and heart, skeletal muscle, pancreas, and bone marrow with relatively lower activities.[3] These differences in oxidase activities among different tissues led to the establishment of a complicated network for the pyridoxal 5′-P distribution because tissues with high oxidase activities produce pyridoxal 5′-P not only for internal consumption but also for external supply to other tissues with low oxidase activities. An example of the distribution network suggested by Lumeng et al.[4] is that the synthesis of pyridoxal 5′-P in muscle is not adequate for its own tissue consumption and as a result, additional supply of pyridoxal 5′-P has to come from either the liver cells or erythrocytes via the circulation. Each tissue maintains an independent pool of vitamin B_6 which includes pyridoxine, pyridoxamine, and pyridoxal. The content of different chemical forms of vitamin B_6 in this pool is regulated by a combination of enzymes, such as pyridoxine 5′-P oxidase, pyridoxal kinase, different species of phosphatases, and various pyridoxal 5′-binding proteins. Then, the activity of any one of the above enzymes or proteins is also regulated by metabolites from other metabolic pathways. This is demonstrated by findings such as decreases in pyridoxine 5′-P oxidase activity as a result of riboflavin deficiency[5] and the activation of rat liver oxidase activity by 3-hydroxykynurenine and 3-hydroxyanthranilate both of which are metabolites of tryptophan metabolism.[6]

Apart from mammalian tissues, pyridoxine 5′-P oxidase activities have also been found in other eukaryotic systems, including yeast[7] and wheat seedlings.[8] The yeast enzyme has been found to be activated by various aliphatic amines[7] and no isoenzymes from yeast have been found. Three isoenzyme forms of pyridoxine 5′-P oxidase have been identified from wheat seedlings and two of them have been partially purified.[8]

Although much evidence has shown that pyridoxine 5′-P oxidase is an enzyme widely distributed in tissues because of its importance in biochemical reactions in both mammalian and plant tissues, it appears that tumor tissue utilizes a different pathway in the synthesis of pyridoxal 5′-P apart from the pathway utilized by normal tissues. Nutter et al.[9] reported that no pyridoxine 5′-P oxidase activity was detected in Morris hepatoma cells suggesting

that tumor tissues do not require the oxidase activity for pyridoxal 5′-P synthesis. The possibilities of acquiring the vitamin from other normal tissues by tumor tissues or synthesizing it via a nonconventional pathway have been suggested.

II. ASSAY

The optimum pH of pyridoxine (pyridoxamine) 5′-P oxidase was recorded at 9 for the rabbit liver enzyme and 8.4 for the brain enzyme. Several assay systems have been designed for the oxidase.

A. COLORIMETRIC METHOD

The colorimetric method as developed by Wada and Snell[2] utilizes the development of a color adduct from the reaction between phenylhydrazine in sulfuric acid with pyridoxal 5′-P. The formation of a yellowish complex can be monitored spectrophotometrically at 412 nm. An extinction coefficient of 23,000 $cm^{-1}\ M^{-1}$ is used for calculating the concentration of the complex which is directly proportional to enzymatic activity. This method is more appropriately adopted for the assay of oxidase activity in crude homogenate because of interference created by turbidity of the assay mixture. Prior to the addition of phenylhydrazine and further colorimetric measurement, the proteins in the assay mixture should have been precipitated using 1*M* trichloroacetic acid and then removed by centrifugation.

B. SPECTROPHOTOMETRIC METHOD

This spectrophotometric method is found to be more convenient for routine procedures. It measures the oxidase activity by monitoring the formation of pyridoxal 5′-P which is proportional to the increase in absorbance at 388 nm. Pyridoxal 5′-P is known to have an extinction coefficient of 4900 $cm^{-1}\ M^{-1}$ at pH 7. Initial rate measurement is carried out by monitoring the change in absorbance at 388 nm for at least 3 min in a spectrophotometer. This method is only recommended for the assay of pyridoxine 5′-P oxidase activity in nonturbid solution.

C. OTHER METHODS

Other less commonly used methods include continuous monitoring using polarographic techniques for measurement for O_2 consumption or using substrates containing either a fluorescent or radioactive group that is released by the oxidase reaction.[10]

For the assay of rabbit liver pyridoxamine 5′-P oxidase, Kazarinoff and McCormick[11] use 0.2 *M* Tris-HCl buffer at pH 8 as the assay medium. In addition, pyridoxamine 5′-P was the preferred substrate used although no difference in the maximum velocity between pyridoxamine 5′-P or pyridoxine 5′-P was observed. However, a difference of tenfold in oxidase activity was observed between using the two phosphorylated vitamins as substrates in the assay of the brain enzyme. Therefore, Kwok and Churchich[12] recommended the use of pyridoxine 5′-P as the sensitive substrate in the assay of pyridoxine 5′-P oxidase activity in brain extracts.

III. PURIFICATION OF PYRIDOXINE 5′-P OXIDASE

Pyridoxine 5′-P oxidase was purified from rabbit liver by Kazarinoff and McCormick[11] and from porcine brain by Kwok and Churchich.[12] The inclusion of a step using phosphopyridoxyl-Sepharose by Churchich[13] led to a higher efficiency achieved in the purification of pig brain oxidase and later, this step of affinity chromatographic procedure was adapted successfully in the purification of other mammalian oxidases.[14,15]

TABLE 1
Purification of Pyridoxine-5-Phosphate Oxidase from Sheep Brain

	Volume (ml)	Protein (mg)	Specific activity (U/mg)	Total activity (U)
Homogenate	28379	752040	0.06	50,019
$(NH_4)_2SO_4$ (40—60%)	2277	81070	0.155	12,614
DEAE-cellulose fraction	2152	10440	1.29	13,560
pH 5.0 treatment	2150	3250	4.2	13,500
Phosphopyridoxyl-Sepharose	500	25	312	7,800
Sephadex G-100	22	15.3	341	5,226

Note: The purification of pyridoxine 5′-P oxidase was made from 20 kg of sheep brain.

TABLE 2
Purification of Pyridoxal Kinase from a Sheep Brain

Treatment	Volume (ml)	Protein (mg)	Protein (mg/ml)	Specific activity (units/mg)	Total activity (units)
Homogenate 40—60%	10500	278250	26.5	0.125	34781
$(NH_4)_2SO_4$ fraction	1680	59808	35.6	0.668	39951
DEAE-cellulose fraction	1500	9450	6.3	5.81	54904
Pyridoxal-agarose fraction	60	64	1.07	5.32	34048
Sephadex G-100	40	25.7	0.643	1104	28373

Note: The purification of pyridoxal kinase was made from 20 kg of sheep brain tissue wet weight.

A. SHEEP BRAIN PYRIDOXINE 5′-P OXIDASE

Purification of pyridoxine 5′-P oxidase from sheep brain is adapted from the procedure for the purification of pyridoxal kinase.[16] Both procedures share the common steps of homogenization, ammonium sulfate fractionation, and DEAE-cellulose chromatography. During the elution of DEAE-cellulose chromatography, the profile exhibits partially overlapping peaks of kinase and oxidase activities. Fractions containing both enzymatic activities are allowed to pass through a pyridoxal-Sepharose column which retains only the kinase activity, and the straight-through material is combined with other fractions containing the oxidase activity. Acid precipitation using 0.2 *N* acetic acid to adjust the pH from 7.4 to 3.5 and phosphopyridoxyl-Sepharose chromatography eluted with 10^{-2} *M* pyridoxal 5′-P at pH 5 are introduced as further steps of the purification procedure after DEAE-cellulose chromatography. The last step of gel filtration using Sephadex G-100 provides the separation of the oxidase from both contaminating proteins and also the eluting ligand of the affinity chromatography pyridoxal 5′-P. After the last step of gel filtration, the oxidase preparation is observed as a single protein band in SDS-polyacrylamide gel electrophoresis. Comparison of summarized procedures for the purification of pyridoxine 5′-P oxidase and pyridoxal kinase are shown in Table 1 and 2, respectively.

In 10% SDS-polyacrylamide gel electrophoresis, pyridoxine 5′-P oxidase was reported as a protein component of 30 kDa in molecular mass. However, in 10 to 20% gradient polyacrylamide gel electrophoresis, protein components characterized by molecular masses of 60 kDa, 90 kDa, and 120 kDa, were detected by staining with Coomassie dye for enzymatic activity (Figure 1). The 60 kDa protein band has appeared consistently as the major species among various molecular mass components but the band intensities of other higher molecular mass components vary from preparation to preparation. Results suggest that the existence of higher molecular mass species pyridoxine 5′-P oxidase may be due to aggregation.

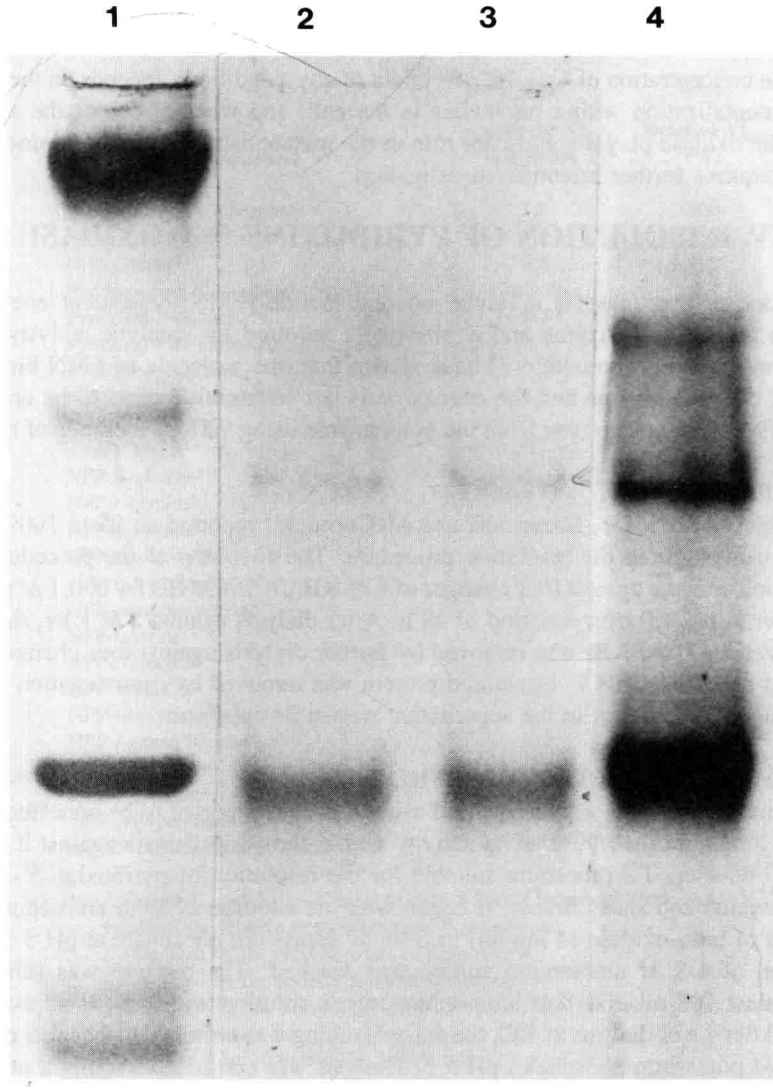


FIGURE 1. 10 to 20% gradient polyacrylamide gel electrophoresis, Lane 1 represents molecular weight markers = IgG, 160,000; aspartate aminotransferase, 110,000; bovine serum albumin, 68,000; and ovalalbumin, 43,000. Lane 2 and 3 represent pyridoxine 5′-P oxidase stained with Coomassie Blue and lane 4, pyridoxine 5′-P oxidase stained for activity.

B. AGGREGATION OF THE OXIDASE

Visser et al.[12] analyzed the multiple forms of pyridoxine 5′-P oxidase in different pH mediums. At pH 8.4 the high performance liquid chromatography (HPLC) system adapted with the TSK 3000 SW column separated the 60 kDa, and 120 kDa species as separate peaks. As the pH of the elution buffer was reduced from 8.4 to 7.4, the size of the 120 kDa peak became smaller and the 60 kDA peak became larger. Using elution buffer at pH 5.5, the 120 kDa peak completely disappeared from the profile and the 60 kDa peak was the sole peak detected. By quantitating the integrated peak size of the 120 kDa and 60 kDa component at various concentrations of proteins a dissociation constant of $4 \times 10^{-5}\ M$ was calculated for the association process: 2 dimer $\rightleftarrows$ tetramer at pH 7.4. The above results obtained by Visser et al.[17] clearly indicate that the aggregation process undergoing pyridoxine 5′-P oxidase is reversible and predominantly pH dependent. This implies that at pH 7.4,

which is the physiological pH of mammalian body system, the oxidase is able to aggregate if the concentration of the enzyme is above 4×10^{-5} *M*. Whether pyridoxine 5′-P oxidase can reach the concentration of 4×10^{-5} *M* under *in vivo* conditions depends on the existence of compartmentalization within organelles in the cell; and whether or not the aggregated species of the oxidase play a significant role in the metabolism of vitamin B_6 under *in vivo* conditions requires further scientific investigation.

IV. RESOLUTION OF PYRIDOXINE 5′-P OXIDASE

Pyridoxine 5′-P oxidase is a flavin mononucleotide (FMN)-dependent enzyme. The FMN group acts as a coenzyme and is absolutely required for catalytic activity. Purified enzymes from different sources[11,13,15] have shown that one molecule of FMN binds to one molecule of dimeric enzyme and the coenzyme is not covalently bound to be apoenzyme. Therefore, FMN can be removed from the holoenzyme using various methods of resolution.

A. THE KBr METHOD

For rabbit liver oxidase, Kazarinoff and McCormick[11] reported an 85 to 100% yield of apoenzyme using KBr in the resolution procedure. The first step of the procedure was to dialyze the holoenzyme against four changes of 2 *M* KBr, 0.1 m*M* EDTA in 0.1 *M* potassium acetate buffer at pH 4.0 over a period of 48 h. After dialysis against 2 *M* KBr, the pH was then readjusted to 7 and KBr was removed by further dialysis against four changes of 0.02 *M* potassium phosphate, pH 7. Denatured protein was removed by centrifugation at 18,000 Xg for 10 min. The oxidase in the supernatant was in its apo-form.

B. THE AMMONIUM SULFATE METHOD

For the oxidase from brain, the method using KBr was reported to be noneffective since the enzyme lost more than 90% of its activity within the 48-h dialysis against 2 *M* KBr.[18] Choi et al.[15] developed a procedure suitable for the resolution of pyridoxine 5′-P oxidase from both porcine and sheep brains. It began with the addition of solid ammonium sulfate to a solution of holo-oxidase (4 mg/ml) in 0.01 *M* potassium phosphate at pH 5.5. A final concentration of 1.8 *M* ammonium sulfate was reached. The mixture was subsequently dialyzed against 100 ml of 1.8 *M* ammonium sulfate solution with the pH adjusted to 3.5 using HCl. After 4 h of dialysis at 4°C, the dialysis tubing was switched to a beaker containing 2 l of 0.01 *M* potassium phosphate, pH 5.5. Dialysis was continued overnight at 4°C with one change of buffer. The end result of this procedure accounted for 95% resolution and 1% loss of the initial oxidase activity after reconstitution with FMN.

V. SUBSTRATE SPECIFICITIES

Pyridoxine (pyridoxamine) 5′-P catalyzes the oxidation of pyridoxine 5′-P, pyridoxamine 5′-P and *N*-(phosphopyridoxyl) amines. As a substrate of the oxidase, a compound requires the presence of a phosphate group. No oxidation of the unphosphorylated form of substrates or substrate analogues catalyzed by pyridoxine 5′-P oxidase has been observed. Kazarinoff et al.[11] summarized the substrate specificities of pyridoxine 5′-P oxidase from rabbit liver in Table 3.

Relative maximum velocities using pyridoxine 5′-P and pyridoxamine 5′-P as substrates were found to be the same for the liver enzyme. However, pyridoxine 5′-P was found to be tenfold more active than pyridoxamine 5′-P for the brain oxidase. For phosphopyridoxyl derivatives, the insertion of an $-NO_2$ group into the para-position of the aromatic carboxylic acids attached to the phosphopyridoxyl moiety yields a good substrate for the oxidase whereas an OH inserted into the para-position diminishes the K_{cat} value (Table 4). It appears that

TABLE 3
Activity of Substrate Analogues in Pyridoxamine-P Oxidase System

Compound	Apparent K_M^a ($\times 10$ *M*)	Relative V_{max}^a (%)
Pyridoxamine 5′-phosphate	1.0	100
Pyridoxine 5′-phosphate	3.0	100
NPP-glycine[b]	6.8	100
Pyridoxamine 5′-sulphate[c]	Not active	
Pyridoxal-P-oxime[d]	2.1	
Pyridoxal-P-*O*-carboxymethyloxime[d]	2.5	
5′-Homopyridoxine-P[e]	0.59	10
5′-Methylpyridoxine-P[e]	3.1	60
α-HPP-ornithine[f]	53	100
α-NPP-lysine[f]	20	20
NPP-B-alanine[g]	11	85
NPP-L-alanine[g]	22	140
NPP-D-alanine[g]	77	130
NPP-L-α-aminobutyrate[g]	9.1	96
NPP-D-α-aminobutyrate[g]	29	120
NPP-α-aminobutyrate[g]	77	130
NPP-L-serine[g]	13	39
NPP-L-leucine[g]	7.5	86
NPP-D-leucine[g]	12.5	120
NPP-benzylamine[g]	3.3	120
NPP-L-phenylalanine[g]	9.5	54
NPP-L-tyrosine[g]	3.1	57
NPP-D-tyrosine[g]	160	55
NPP-L-tyrptophan[g]	12	18
α-NPP-diaminodecane[h]	40	20

[a] Apparent K_M nd V_{max} values determined from Lineweaver-Burk plots. V_{max} values given relative to pyridoxamine-P as substrate.
[b] Synthesized by the method given in Reference 4.
[c] Synthesized by the method given in Reference 19.
[d] Synthesized by the method given in Reference 20; the value given is the inhibition constant K_i.
[e] Data taken from Reference 3.
[f] These compounds were kindly provided by Dr. James K. Coward, Department of Pharmacology, Yale University School of Medicine, New Haven, Conn.
[g] Data taken from Reference 4.
[h] Synthesized by the method given in Reference 21.

From Kazarinoff, M. N. and McCormick, D. B., *J. Biol. Chem.*, 250, 3436, 1975. With permission.

substituents affect the reactivity by their ability to withdraw electrons from the reaction site. Churchich[13] showed that substituents with positive σ values (–COOH and $-NO_2$) increase the reactivity of the substrate and substituents with negative σ values (–OH) tend to decrease the reactivity of phosphopyridoxyl moiety with the oxidase. Gregory[19] reported that *N*-acetyl-phosphopyridoxyl-lysine was the best substrate for the liver oxidase. Using it as a substrate, the oxidase exhibited 75% higher maximum activity than pyridoxamine 5′-P (Table 5). However, its usage as a substrate for the brain enzyme has never been tested.

VI. PROPERTIES OF THE FMN-BINDING SITE

A. STUDIES USING FMN ANALOGUES

Various analogues have been used by Kazarinoff and McCormick[20] to study the coenzyme