EDMONDSON, Dale Edward, 1942-
PROTEIN-FLAVIN INTERACTIONS IN THE SHETHNA
FLAVOPROTEIN FROM AZOTOBACTER.

University of Arizona, Ph.D., 1970
Chemistry, biological

University Microfilms, A XEROX Company, Ann Arbor, Michigan
PROTEIN-FLAVIN INTERACTIONS IN THE SHETHNA
FLAVOPROTEIN FROM AZOTOBACTER

by
Dale Edward Edmondson

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF CHEMISTRY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1970
I hereby recommend that this dissertation prepared under my direction by DALE EDWARD EDMONDSON entitled PROTEIN-FLAVIN INTERACTIONS IN THE SHEATHNA FLAVOPROTEIN FROM AZOTOBACTER be accepted as fulfilling the dissertation requirement of the degree of DOCTOR OF PHILOSOPHY.

Dissertation Director April 24, 1970

After inspection of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:

*This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Dale C. Edmondson
ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. Gordon Tollin, whose direction and inspiration made this dissertation possible. Appreciation is also extended to Dr. Jimmy W. Hinkson for his introduction to this area of research.

The contributions of flavin analogs and flavoenzymes from Dr. V. Massey, Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan, Dr. D. B. McCormick, Division of Biological Sciences, Cornell University, Ithaca, New York, Dr. P. Hemmerich, University of Konstanz, Konstanz, Germany, and Dr. M. Cusanovich of this Department are deeply appreciated. The assistance of Dr. G. Adams and of P. Adams in performing the ultracentrifuge experiments is gratefully acknowledged.

The receipt of Public Health Service Predoctoral Fellowship, 5-F01-GM-37, 799-01 to 03, from the National Institute of General Medical Sciences from 1967 to 1970 is most gratefully acknowledged.

The encouragement from my family during these past years is especially appreciated.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiv</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Flavin Chemistry</td>
<td>2</td>
</tr>
<tr>
<td>Structure and Nomenclature</td>
<td>2</td>
</tr>
<tr>
<td>Properties of the Oxidized Flavins</td>
<td>4</td>
</tr>
<tr>
<td>Properties of the Semiquinone Form of Flavins</td>
<td>9</td>
</tr>
<tr>
<td>Properties of the Fully-reduced Flavins</td>
<td>15</td>
</tr>
<tr>
<td>Spectroscopy of Oxidized Flavins</td>
<td>18</td>
</tr>
<tr>
<td>Flavin-protein Interactions</td>
<td>20</td>
</tr>
<tr>
<td>Experimental Approaches</td>
<td>20</td>
</tr>
<tr>
<td>Protein Complexes of Isoalloxazine Ring-modified Flavins</td>
<td>22</td>
</tr>
<tr>
<td>Protein Complexes of Side-chain Modified Flavins</td>
<td>24</td>
</tr>
<tr>
<td>Possible Protein Groups Important in Flavin-protein Interactions</td>
<td>27</td>
</tr>
<tr>
<td>The Relation of Redox Activity to Flavoenzyme Properties</td>
<td>29</td>
</tr>
<tr>
<td>Summary</td>
<td>33</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>35</td>
</tr>
<tr>
<td>Materials</td>
<td>35</td>
</tr>
<tr>
<td>Flavoenzymes</td>
<td>35</td>
</tr>
<tr>
<td>Flavin Analogs</td>
<td>36</td>
</tr>
<tr>
<td>Other Materials</td>
<td>38</td>
</tr>
<tr>
<td>Instruments</td>
<td>39</td>
</tr>
</tbody>
</table>

iv
TABLE OF CONTENTS—Continued

Experimental Procedures ........................................ 41

Maintenance and Growth of *Azotobacter vinelandii* Cultures ........ 41
Isolation of the Shethna Flavoprotein ........................... 43
Preparation of the Apoprotein .................................. 45
Circular Dichroism Measurements ............................... 47
Analytical Ultracentrifugation Experiments .................... 49
Amino Acid Composition ....................................... 51
Tyrosine Spectrophotometric Titration ........................ 51
Determination of Binding Constants with a Number of Flavin Analogs .......................... 52
Preparation of Semiquinone Form of Flavoenzymes ............... 54

RESULTS ..................................................................... 57

Circular Dichroism Studies of the Flavin Chromophore .......... 57
CD and Absorption Spectra of Unbound Flavins .................. 59
CD and Absorption Spectra of Protein-bound Flavins ......... 68
A Comparison of the Circular Dichroism Spectra of a Number of Flavoenzymes ......................... 80
CD and Absorption Spectra of the Oxidized Forms ............. 80
CD and Absorption Spectra of the Semiquinone Forms of Flavoenzymes ............................ 93

Chemical and Physical Properties of the Shethna Flavoprotein 107
Molecular Weight ............................................... 107
Amino Acid Composition ...................................... 113

Resolution of the Shethna Flavoprotein ......................... 116
The Binding of Flavin Analogs to the Shethna Apoprotein .. 123
Equilibrium Binding ......................................... 123
Fluorescence Quenching of the Flavin Analogs upon Binding . 129
CD Spectra of Protein-bound Riboflavin Analogs .............. 130
Kinetics of Flavin Binding to the Apoprotein ................. 135
# TABLE OF CONTENTS—Continued

| Properties of Semiquinones of Flavoenzymes and FAD-analog Complexes of the Shethna Apoprotein | 138 |
| Photochemical Reduction | 138 |
| Chemical Reduction by Dithionite | 143 |
| Oxidation by Molecular Oxygen | 152 |
| Near Ultraviolet Spectral Studies of Protein-bound Flavins | 158 |
| Near UV Absorption Spectra of Oxidized and Semiquinone Flavoproteins | 158 |
| Near UV CD Spectra of Oxidized and Semiquinone Flavoproteins | 165 |
| Effect of pH on Semiquinone Oxidation by Oxygen. | 173 |
| The Relation between Flavin Redox Form and Protein Conformation in the Shethna Flavoprotein | 180 |
| Circular Dichroism | 180 |
| Proton Magnetic Resonance | 181 |
| DISCUSSION | 184 |
| Flavoenzyme Classification | 184 |
| Dithionite Reduction of the Shethna Flavoprotein Semiquinone | 185 |
| Oxidation of the Shethna Flavoprotein Semiquinone by Oxygen | 187 |
| Flavin Binding to the Shethna Apoprotein | 189 |
| REFERENCES | 199 |
## LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Absorption Spectra of Neutral and Anionic Flavin Semiquinones</td>
<td>12</td>
</tr>
<tr>
<td>2. Postulated Mechanism for Oxygen Oxidation of Hydroquinone Form of Oxidases and Dehydrogenases</td>
<td>32</td>
</tr>
<tr>
<td>3. Absorption at 280 nm as a Function of Shethna Apoprotein Concentration</td>
<td>46</td>
</tr>
<tr>
<td>4. Resolved Absorption and CD Spectra of Tetra-O-acetyl Riboflavin in 1, 2-dichloroethane</td>
<td>60</td>
</tr>
<tr>
<td>5. Resolved Absorption and CD Spectra of Tetra-O-acetyl Riboflavin in 0.025M Phosphate Buffer (pH = 7.0)</td>
<td>61</td>
</tr>
<tr>
<td>6. Resolved Absorption and CD Spectra of Riboflavin in 0.1M Phosphate Buffer (pH = 7.0)</td>
<td>62</td>
</tr>
<tr>
<td>7. Resolved Absorption and CD Spectra of FMN in 0.1M Phosphate Buffer (pH = 7.0)</td>
<td>63</td>
</tr>
<tr>
<td>8. Resolved CD and Absorption Spectra of the Shethna Flavoprotein</td>
<td>70</td>
</tr>
<tr>
<td>9. Resolved Absorption and CD Spectra of <em>C. pasteurianum</em> Flavodoxin</td>
<td>71</td>
</tr>
<tr>
<td>10. Resolved CD and Absorption Spectra of the DeoxyFMN-Shethna Apoprotein Complex</td>
<td>72</td>
</tr>
<tr>
<td>11. Difference Spectra of Shethna Apoprotein-bound Flavin Minus Unbound Flavin</td>
<td>75</td>
</tr>
<tr>
<td>12. CD Spectra of Flavin Solutions in the Near Ultraviolet Region</td>
<td>79</td>
</tr>
<tr>
<td>13. Resolved CD and Absorption Spectra of <em>P. elsdenii</em> Flavodoxin in the Visible Region</td>
<td>81</td>
</tr>
<tr>
<td>Page</td>
<td>Illustration Description</td>
</tr>
<tr>
<td>------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>82</td>
<td>14. Resolved CD and Absorption Spectra of R. rubrum Flavodoxin in the Visible Region</td>
</tr>
<tr>
<td>84</td>
<td>15. Resolved Absorption and CD Spectra of Spinach Ferredoxin-TPNH Reductase in the Visible Region</td>
</tr>
<tr>
<td>85</td>
<td>16. Resolved CD and Absorption Spectra of Glucose Oxidase in the Visible Region</td>
</tr>
<tr>
<td>86</td>
<td>17. Resolved CD and Absorption Spectra of Snake Venom L-aminio Acid Oxidase (C. adamanteus) in the Visible Region</td>
</tr>
<tr>
<td>90</td>
<td>18. Resolved CD and Absorption Spectra of the 3-methylFMN-Shethna Apoprotein Complex in the Visible Region</td>
</tr>
<tr>
<td>92</td>
<td>19. CD and Absorption Spectra of the IsoFMN-Shethna Apoprotein Complex in the Visible Region</td>
</tr>
<tr>
<td>94</td>
<td>20. Absorption and CD Spectra of the Semiquinone Form of the Shethna Flavoprotein in the Visible Region</td>
</tr>
<tr>
<td>95</td>
<td>21. Absorption and CD Spectra of the Semiquinone Form of R. rubrum Flavodoxin in the Visible Region</td>
</tr>
<tr>
<td>96</td>
<td>22. CD and Absorption Spectra of the Semiquinone Form of the 3-methylFMN-Shethna Apoprotein Complex in the Visible Region</td>
</tr>
<tr>
<td>97</td>
<td>23. CD and Absorption Spectra of the Semiquinone Form of the DeoxyFMN-Shethna Apoprotein Complex in the Visible Region</td>
</tr>
<tr>
<td>98</td>
<td>24. CD and Absorption Spectra of the Semiquinone Form of the IsoFMN-Shethna Apoprotein Complex in the Visible Region</td>
</tr>
<tr>
<td>No.</td>
<td>Illustration Description</td>
</tr>
<tr>
<td>------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>25.</td>
<td>CD and Absorption Spectra of the Semiquinone Form of <em>C. pasteurianum</em> Flavodoxin in the Visible Region</td>
</tr>
<tr>
<td>26.</td>
<td>CD and Absorption Spectra of the Semiquinone Form of <em>P. elsdenii</em> Flavodoxin in the Visible Region</td>
</tr>
<tr>
<td>27.</td>
<td>CD and Absorption Spectra of the Neutral Semiquinone Form of Glucose Oxidase in the Visible Region</td>
</tr>
<tr>
<td>28.</td>
<td>CD and Absorption Spectra of the Anionic Semiquinone Form of Glucose Oxidase in the Visible Region</td>
</tr>
<tr>
<td>29.</td>
<td>CD and Absorption Spectra of the Anionic Semiquinone Form of (<em>C. adamanteus</em>) L-amino Acid Oxidase in the Visible Region</td>
</tr>
<tr>
<td>30.</td>
<td>Sedimentation of the Shethna Flavoprotein as a Function of Time</td>
</tr>
<tr>
<td>31.</td>
<td>Plot of $A^2/H^2$ versus Time in the Determination of the Diffusion Coefficient of the Shethna Flavoprotein</td>
</tr>
<tr>
<td>32.</td>
<td>The Variation of the Diffusion Coefficient of the Shethna Flavoprotein with Concentration</td>
</tr>
<tr>
<td>33.</td>
<td>Plot of $\ln y/r$ versus $r^2$ in the Determination of the Molecular Weight of the Shethna Flavoprotein by Sedimentation Equilibrium</td>
</tr>
<tr>
<td>34.</td>
<td>Sephadex G-100 Column Chromatography of the Shethna Flavoprotein and Apoprotein</td>
</tr>
<tr>
<td>35.</td>
<td>Near Ultraviolet CD and Absorption Spectra of the Shethna Apoprotein</td>
</tr>
<tr>
<td>36.</td>
<td>Far Ultraviolet CD Spectra of the Shethna Flavoprotein, Apoprotein and Reconstituted Flavoprotein</td>
</tr>
<tr>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td></td>
</tr>
<tr>
<td>124</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td></td>
</tr>
<tr>
<td>131</td>
<td></td>
</tr>
<tr>
<td>132</td>
<td></td>
</tr>
<tr>
<td>134</td>
<td></td>
</tr>
<tr>
<td>136</td>
<td></td>
</tr>
<tr>
<td>141</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td></td>
</tr>
<tr>
<td>146</td>
<td></td>
</tr>
<tr>
<td>147</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td></td>
</tr>
<tr>
<td>153</td>
<td></td>
</tr>
<tr>
<td>159</td>
<td></td>
</tr>
<tr>
<td>Illustration</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>50</td>
<td>Ultraviolet Spectra of Protein-bound and Free FMN Analogs</td>
</tr>
<tr>
<td>51</td>
<td>Resolved Ultraviolet Difference Spectra of Semiquinone FMN Analog-Shethna Apoprotein Complexes Minus Oxidized Forms</td>
</tr>
<tr>
<td>52</td>
<td>Relation between Semiquinone Absorptivity in the UV and Oxygen Reactivity</td>
</tr>
<tr>
<td>53</td>
<td>Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of FMN, 3-methylFMN and IsoFMN-Shethna Apoprotein Complexes</td>
</tr>
<tr>
<td>54</td>
<td>Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of the DeoxyFMN-Shethna Apoprotein Complex</td>
</tr>
<tr>
<td>55</td>
<td>Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of the 2-thioFMN-Shethna Apoprotein Complex</td>
</tr>
<tr>
<td>56</td>
<td>Ultraviolet Circular Dichroism Spectra of P. elsdenii Flavodoxin in the Oxidized and Semiquinone Forms</td>
</tr>
<tr>
<td>57</td>
<td>Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of C. pasteurianum Flavodoxin</td>
</tr>
<tr>
<td>58</td>
<td>Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of R. rubrum Flavodoxin</td>
</tr>
<tr>
<td>59</td>
<td>The Influence of pH on the Apparent First-order Rate Constant for Oxidation of the Shethna Flavoprotein Semiquinone by Oxygen</td>
</tr>
<tr>
<td>60</td>
<td>The Relation between the Reciprocal of the Apparent First-order Rate Constant for Semiquinone Oxidation by Oxygen and Hydrogen Ion Concentration</td>
</tr>
<tr>
<td>61</td>
<td>A Comparison of the Rate of Shethna Flavoprotein Semiquinone Disproportionation and Oxygen Oxidation at pH = 11.0</td>
</tr>
<tr>
<td>Illustration</td>
<td>Title</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>62.</td>
<td>Tyrosyl Ionization in the Oxidized and Semiquinone Forms of the Shethna Flavoprotein and in the Shethna Apoprotein</td>
</tr>
<tr>
<td>63.</td>
<td>The Effect of Redox Form on the 220 MHz Proton Magnetic Resonance Spectrum of the Shethna Flavoprotein</td>
</tr>
<tr>
<td>64.</td>
<td>Proposed Configuration of the FMN Molecule in Relation to its Binding Site in the Shethna Flavoprotein</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>I. Nomenclature of N(10) Substituted Isoalloxazines</td>
<td>3</td>
</tr>
<tr>
<td>II. Molar Extinction Coefficients of Flavoenzymes</td>
<td>37</td>
</tr>
<tr>
<td>III. Spectral Properties of Flavin Analogs</td>
<td>38</td>
</tr>
<tr>
<td>IV. Composition of Burk's Media with Carbon Source</td>
<td>42</td>
</tr>
<tr>
<td>V. The Resolved Spectral Bands of Free and Protein-bound Flavins</td>
<td>66</td>
</tr>
<tr>
<td>VI. Intensities of the Resolved Spectral Bands of Free and Protein-bound Flavin</td>
<td>73</td>
</tr>
<tr>
<td>VII. The Resolved Spectral Bands of the Dehydrogenases and Oxidases</td>
<td>88</td>
</tr>
<tr>
<td>VIII. Intensities of the Resolved Spectral Bands of the Dehydrogenases and Oxidases</td>
<td>89</td>
</tr>
<tr>
<td>IX. Sedimentation Coefficient of Shethna Flavoprotein of Different Concentrations</td>
<td>107</td>
</tr>
<tr>
<td>X. The Amino Acid Composition of the Shethna Flavoprotein</td>
<td>114</td>
</tr>
<tr>
<td>XI. Equilibrium Binding Constants of Flavin Analogs to the Shethna Apoprotein</td>
<td>126</td>
</tr>
<tr>
<td>XII. Second-order Rate Constants for Flavin Binding to the Shethna Apoprotein</td>
<td>137</td>
</tr>
<tr>
<td>XIII. Photochemical Semiquinone Production and Chemical Reduction of Flavoenzymes and FAD Analogs at pH = 7.0</td>
<td>140</td>
</tr>
<tr>
<td>XIV. Oxygen Oxidation of the Neutral Semiquinone Forms of Flavoenzymes and FMN Analog- Shethna Apoprotein Complexes</td>
<td>154</td>
</tr>
</tbody>
</table>
ABSTRACT

The interactions between protein and flavin in flavoenzymes have been investigated using the Shethna flavoprotein from *Azotobacter vinelandii* as a model system. The flavoprotein has a molecular weight of 23,000 g-mole$^{-1}$ as determined in the analytical ultracentrifuge, and has an amino acid composition similar to the lower molecular weight flavodoxins from *C. pasteurianum* and *P. elsdenii*. Circular dichroism spectra indicate a low amount of alpha helix and the presence of beta structure in the flavoprotein. No major changes in protein conformation were observed upon reducing the FMN prosthetic group to the semiquinone or to the hydroquinone form.

The reversible resolution and recombination of FMN with the apoprotein was demonstrated using a variety of spectral and redox properties. The binding, spectral and redox properties of a large number of flavin analog-apoprotein complexes has indicated: (1) the 5' phosphate group is important in binding and is involved in stabilizing the semiquinone form of the protein both directly and by inducing a change in protein geometry about the binding site upon flavin binding; (2) increased interaction between
the side-chain hydroxyl groups and the isoalloxazine ring occurs in the protein-bound flavin; (3) important protein-flavin interactions exist at the 2-carbonyl position, the N(3) position and at the benzenoid portion of the isoalloxazine ring; (4) isoalloxazine ring-protein interactions contribute the major portion of the total binding energy; and (5) the initial and rate-limiting step in the binding process involves the flavin side chain interaction with the protein.

The resistance of the semiquinone form of the Shethna flavoprotein to oxygen oxidation is decreased by a one proton ionization with a $pK \approx 11.5$. The stability of this species has been correlated with the intensity of the 270 nm absorption maximum. The inertness towards oxygen is suggested to be due to the stabilization of the $N(5)$ proton of the neutral flavin semiquinone. The resistance of the semiquinone form at neutral pH to further reduction by dithionite is also diminished by a one proton ionization, $pK \approx 7$. This property seems to be related to the ionization of the hydroquinone form of the flavin.

Absorption, CD, and PMR spectroscopy has indicated the proximity of an aromatic amino acid residue to the FMN in the Shethna flavoprotein. CD data give no evidence for a tryptophan-flavin interaction. The binding of FMN was found to perturb the ionization behavior of the tyrosyl residues.
CD and absorption spectroscopy of model flavin compounds in various solvents, and of several flavoenzymes, indicate three \( \pi \rightarrow \pi^* \) flavin transitions in the 300-500 nm spectral region. No evidence was found for any \( n \rightarrow \pi^* \) transitions. The environment of the flavin group in the flavoenzymes is reflected in the CD spectrum by the relative intensities and signs of the six vibronic bands in this spectral region. The magnitude of the rotational strength of the long-wavelength bands is quite sensitive to the interaction of the side-chain hydroxyl groups with the isoalloxazine ring. Similarities exist in the CD spectra of those flavoenzymes with similar redox function. This provides a direct structural probe for comparing the flavin environments in the dehydrogenases and the oxidases.
INTRODUCTION

Flavoenzymes catalyze a wide variety of oxidation-reduction reactions in living cells (Mahler and Cordes, 1966; Dixon and Webb, 1964). They can accept or donate reducing equivalents to molecules as large as proteins (e.g., cytochromes) or as small as oxygen. The flavin coenzyme is particularly versatile in that it can function in either a one or a two electron transfer process. The specificity of a flavoenzyme for a particular substrate(s) and for a particular mode of redox behavior can be considered to arise from protein groups oriented in the vicinity of the flavin and to the specific interactions of the protein moiety with the flavin molecule.

Additional specificity and complexity can result from the presence of other redox active groups in the flavoenzyme. Iron and/or molybdenum are functionally active in metalloflavoenzymes such as xanthine oxidase (Rajagopalan and Handler, 1968). A heme group may also function along with flavin in catalysis as is found in cytochrome b₂ (Hiromi and Sturtevant, 1968). A redox active disulfide bond has been implicated in the catalytic activities of lipoic acid dehydrogenase and glutathione reductase (Palmer and Massey, 1968).
The study of flavoenzymes as molecular entities has recently been stimulated by the development of more sophisticated techniques in enzyme purification. Advanced instrumentation such as electron spin resonance and circular dichroism spectroscopy have provided tools to probe the structure and to study the catalytic mechanism of flavoenzymes. The recent isolation of low molecular weight flavoenzymes containing only a single flavin group has provided systems particularly amenable to the investigation of protein-flavin interactions.

This study has as its major goal the elucidation of protein interactions with the different redox forms of the flavin and how these affect the properties of the coenzyme. The applicability of the present results to the general field of flavoenzyme chemistry will also be considered.

**Flavin Chemistry**

**Structure and Nomenclature**

The reactive part of the flavin molecule is a heterocyclic, aromatic, three-ring system commonly referred to as the isoalloxazine ring:

![Isoalloxazine Ring Diagram](image)

The numbering system is according to Hemmerich, Veeger, and Wood (1965), and also by Penzer and Radda (1967). The
nature of the R group determines the type of flavin as shown in Table I.

TABLE I

Nomenclature of N(10) Substituted Isoalloxazines

<table>
<thead>
<tr>
<th>R</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-</td>
<td>lumichrome</td>
</tr>
<tr>
<td>CH₃-</td>
<td>lumiflavin</td>
</tr>
<tr>
<td>-CH₂-(CHOH)₃CH₂OH</td>
<td>riboflavin</td>
</tr>
</tbody>
</table>

The flavin coenzyme exists in only two forms in all of the flavoenzymes isolated so far.¹ One of these is the phosphorylated derivative of riboflavin, named flavin mononucleotide (riboflavin-5'-phosphate) and commonly abbreviated as FMN.

![Chemical Structure of FMN]

The second form of the naturally occurring flavin coenzymes is the pyrophosphate ester of FMN and adenosine

¹Succinic dehydrogenase may be an exception (Hemmerich et al., 1969).
monophosphate (AMP) named flavin adenine dinucleotide and abbreviated as FAD:

This coenzyme was first isolated by Warburg and Christian (1933) from the flavoenzyme D-amino acid oxidase.

The flavin molecule can accept either one or two reducing equivalents to form the half-reduced semiquinone and fully-reduced hydroquinone forms. Since either one or both of these redox forms, as well as the oxidized form, may function in catalysis, a discussion of the chemical and physical properties of all three redox forms is important to an understanding of the protein-flavin interactions involved in enzymatic catalysis.

Properties of the Oxidized Flavins

The absorption spectrum of oxidized flavin has four major bands at 445 nm, 373 nm, 266 nm and 222 nm (Beinert, 1960). These bands are quite intense ($\epsilon_M = 10,600 - 32,000$ M$^{-1}$ cm$^{-1}$) and are thought to arise from $\pi \rightarrow \pi^*$ transitions in the aromatic ring system. A more detailed discussion of
the spectroscopy of flavins will be given in a later section.

The effect of substituents on the isoalloxazine ring with regard to absorption spectra has been studied by Dudley et al. (1964). Penzer and Radda (1967) have also compiled a similar listing from examples in the literature. The results indicate no appreciable spectral shifts upon alkylation of the N(3) position. Removal of the alkyl group at N(10) causes a blue shift of approximately 70 nm. (Derivatives of this type are called alloxazines.) The absorption spectrum is also quite sensitive to alkylation at N(1). These latter compounds have spectral maxima at 450, 378, 264, and 222 nm. Removal of the methyl group from the 7-position and methylation of the 5-position (isoflavin) results in a bathochromic shift in the 373 nm transition and a 50% decrease in extinction of the 446 nm band. The substitution of sulfur for oxygen in the 2- or the 4-position carbonyls results in a red shift of the 445 nm transition to approximately 490 nm. Bands at 390 nm, 316 nm, and 268 nm are also observed in the spectrum of the 2-thio flavin. It is reasonable to expect that these bands correlate with the four normal flavin bands at 445, 375, 266, and 222 nm. No reliable spectral measurements below 250 nm are available for the thio derivatives. The increased polarizability of the sulfur atom relative to the oxygen atom is probably responsible for these large spectral shifts.
The optical activity of the flavin absorption bands is due to the presence of an asymmetric side-chain such as is present in riboflavin or FMN. Lumiflavin has no natural optical activity (Tollin, 1968a) inasmuch as the isoalloxazine ring itself has no asymmetric carbons. This induced optical activity can best be described as a static perturbation (Schellman, 1966) rather than a coupling of the electronic transitions of the ribityl chain with the isoalloxazine ring (Eyring, Liu, and Caldwell, 1968). This is because of the large energy separation between the isoalloxazine electronic transitions and the ribityl transitions which would be expected to lead to a small degree of coupling. FAD has a circular dichroism (CD) spectrum entirely different from that of FMN or riboflavin (Miles and Urry, 1968). The reasons for this will be discussed below.

The oxidized flavin ring is amphoteric, with pK's of protonation and ionization at ≈0 and 10.4, respectively (Hemmerich et al., 1965). The loss of a proton (from the N/3 position) results in a small bathochromic shift (Penzer and Radda, 1967). The site of protonation is at N(1), inasmuch as the absorption spectrum of protonated flavin is similar to flavin analogs with an alkyl bridge placed between N(1) and N(10) (Dudley et al., 1964). Furthermore, circular dichroism and magnetic circular dichroism studies on isoalloxazines and quinoxaline in acid solution also suggest N(1) as the site of protonation.
(Tollin, 1968a). The structures of the ionic forms of oxidized flavin are:

Flavins have an intense fluorescence with an emission maximum at about 530 nm. With the exception of a few flavoenzymes, e.g., lipoyl dehydrogenase (Palmer and Massey, 1968), the flavin fluorescence is completely quenched upon binding to protein. The anionic and cationic flavin species are not fluorescent. Most substituted flavins are fluorescent with emission maxima approximately the same as that of riboflavin. An exception is the 2-thio flavins, which are not fluorescent (Mitchell and Hastings, 1969). Efficient quenchers of flavin fluorescence are electrolytes, e.g., KI, complexing aromatic substances such as phenols and indoles, metal ions, and thiols (Penzer and Radda, 1967). FAD has a fluorescence quantum yield of about 0.05 in aqueous solution (Weber, 1950), which is only one-fifth that of FMN. (See below.)

Koziol (1969) has studied the fluorescence properties of flavins in various solvents. The fluorescence quantum yields of lumiflavin and tetra-O-butyryl riboflavin are significantly higher than that of riboflavin. The
difference in fluorescence quantum yields are greatest in non-polar solvents. In polar solvents, such as water or acetic acid, lumiflavin has only a slightly higher quantum yield than riboflavin. This suggests that the side-chain hydroxyl groups act in dissipating singlet excited state energy in a non-radiative manner, and that the ring-side chain interactions are highly solvent dependent. This latter property is also indicated by CD spectra (Tollin, 1968a).

As we have seen, many of the properties of FAD are different from those of either riboflavin or FMN. The available evidence clearly indicates that the isoalloxazine and adenine aromatic rings form an intra-molecular complex in free FAD. The hypochromism in the absorption spectrum (Beinert, 1960), the low fluorescence quantum yield (Weber, 1950), and the electronic coupling of the isoalloxazine and adenine transitions in the CD spectrum (Miles and Urry, 1968) all demonstrate the presence of a complexed form of FAD in aqueous solution. This complex may be broken by lowering the pH, by increasing the temperature, or by the addition of non-polar solvents to an aqueous solution of FAD. Nuclear magnetic resonance (NMR) studies (Kotowycz et al., 1969; Sarma, Dannies, and Kaplan, 1968) also indicate the existence of an equilibrium between a folded and an unfolded FAD conformation. Alkylation of the N(3) position or removal of the ribityl side-chain hydroxyl groups does not have any effect on complex stability (Chassey and McCormick, 1965a).
Recent NMR studies on the flavin amides of tyrosine, tryptophan, and phenylalanine (Föry et al., 1970) indicate a planar "stacked" arrangement of the flavin ring and the aromatic ring of the respective amino acid. As with FAD, the stacking of these flavinyl peptides is dependent upon temperature and solvent. The requirement for water in complex formation indicates the importance of hydrophobic interaction between the two rings.

Properties of the Semiquinone Form of Flavins

The earliest experimental evidence for a semiquinone form of flavin was the observation of a red intermediate by Kuhn and Wagner-Jauregg (1934) upon the reduction of riboflavin with zinc in acid solution, and from the potentiometric titration data of Michaelis and Schwarzzenbach (1938). The observation of absorption bands in the near infrared in samples of 50% reduced flavin by Beinert (1956), and the advent of electron spin resonance (ESR) spectroscopy provided techniques with which to study the semiquinone form of flavin.

Potentiometric titration studies by Michaelis and Schwarzzenbach (1938) indicated that the pK for the ionization of the neutral flavin semiquinone to its anionic form is 6.5. Reinvestigation of the ionization behavior of flavin semiquinones by Draper and Ingrahm (1968) has revised this to 8.3-8.4. This latter pK value has been substantiated by ESR studies (Ehrenberg, Müller, and Hemmerich,
1967) and by spectral studies (Land and Swallow, 1969). ESR studies (Guzzo and Tollin, 1964) have also suggested that the neutral form of the semiquinone is a zwitterion. ESR studies of isotopically substituted flavins (Ehrenberg et al., 1967) have indicated the probable tautomeric form of the anion radical. The main structures of the neutral and anionic semiquinone forms are:

\[ \text{pK} = 8.3 \]

Massey and Palmer (1966) reported the absorption spectra of a number of flavoenzymes in the semiquinone form. On the basis of the interconversion of the semiquinone of glucose oxidase from a species having absorption bands at 600 nm at pH = 6.0, to one having no extinction at that wavelength upon raising the pH to 10.0, they assigned the neutral flavin radical as the blue form and the anionic radical as the red form. Their data indicate a pK = 7.5 for the ionization of the glucose oxidase neutral radical.

Evidence confirming that the spectral properties of the two types of enzyme-bound flavin semiquinones are indeed due to the anionic and neutral forms has come from the work of Ehrenberg et al. (1967) and from the pulse
radiolysis studies of Land and Swallow (1969) on free flavins. The absorption spectra of the neutral and anionic flavin radicals (both free and protein-bound) are given in Figure 1.

Flavin semiquinones may be produced either chemically or photochemically. Dithionite reduction has been used for the production of free flavin semiquinones (Beinert, 1960) and for the production of protein-bound flavin semiquinones (Massey and Palmer, 1966). Due to the complication of sulfite-flavin adduct formation upon dithionite reduction (Swoboda and Massey, 1966), Massey and Palmer (1966) initiated the use of photochemical reduction of flavoproteins to generate the semiquinone form (using EDTA as the electron donor).

The work of Frisell, Chung, and MacKenzie (1959) showed that free flavin was photobleached in the presence of electron donors such as EDTA. The reaction is thought to proceed, with the flavin triplet state \( \text{F}^* \) being the reactive species, in the following manner:

\[
\begin{align*}
\text{F} + \text{h}^\nu &\rightarrow \text{F}^* \\
\text{F}^* &\rightarrow \text{F}_S^* \\
\text{F}_S^* &\rightarrow \text{F}_T^* \\
\text{F}_T^* + \text{electron donor} &\rightarrow \text{FH}^* + \text{photooxidation products}
\end{align*}
\]

In the absence of an electron donor, flavins undergo photobleaching through hydrogen abstraction from the ribityl
Fig. 1. Absorption Spectra of Neutral and Anionic Flavin Semiquinones.

----- Anion riboflavin radical and (——) neutral riboflavin radical (Land and Swallow, 1969)

..... Protein-bound neutral flavin radical of the Shethna flavoprotein (Hinkson and Bulen, 1967)

--- Protein-bound anionic flavin radical of glucose oxidase (Massey and Palmer, 1966)
Fig. 1. Absorption Spectra of Neutral and Anionic Flavin Semiquinones.
side chain and consequent side chain degradation to form lumiflavin or lumichrome (Holmström and Oster, 1961). More recent work has confirmed these ideas (Green and Tollin, 1968; Vaish and Tollin, 1970.

In solutions containing mixtures of oxidized flavin (F) and flavin hydroquinone (FH$_2$), two equilibria are present. The first equilibrium is the dimerization of F and FH$_2$ to form a complex:

$$F + FH_2 \rightleftharpoons (F\cdots FH_2)$$

This dimeric form has a charge-transfer type absorption band at 900 nm (Massey and Palmer, 1962). The second equilibrium present, which is more relevant to this discussion, is the disproportionation of the semiquinone form to the oxidized and fully reduced forms:

$$2FH^* \overset{k_2}{\underset{k-2}{\rightleftharpoons}} F + FH_2$$

Holmström (1964) has determined a rate constant for the neutral FMN semiquinone to be: $k_2 = 3.5 \times 10^8$ M$^{-1}$ sec$^{-1}$. The anionic form of the semiquinone has a similar rate constant of $1 \times 10^8$ M$^{-1}$ sec$^{-1}$ (Holmström, 1964). Land and Swallow (1969) have measured the decay constant of the neutral riboflavin semiquinone ($k_2$) to be $5.7 \times 10^8$ M$^{-1}$ sec$^{-1}$. A similar decay constant ($3.5 \times 10^8$ M$^{-1}$ sec$^{-1}$) was obtained for the anionic riboflavin semiquinone.

Temperature jump kinetic studies (Swinehart, 1965; Barman and Tollin, 1970) support the idea that the neutral
FMN semiquinone originates from the dimer complex of oxidized and hydroquinone flavin (F$_{-}$H$_2$), rather than directly from a bimolecular reaction. The two kinetic constants for the conversion were determined to be: $k_2 = 1.1 \times 10^7$ M$^{-1}$ sec$^{-1}$ and $k_{-2} = 4.8 \times 10^2$ sec$^{-1}$ (Barman and Tollin, 1970). The value for $k_2$ is only in fair agreement with the results of flash photolysis (Holmström, 1964) and pulse radiolysis (Land and Swallow, 1969). The reasons for this are not clear at present.

The rapid disproportionation reaction of the flavin semiquinone makes it very difficult to study its physical and chemical properties. This can be circumvented to a degree by using flash photolysis techniques (Vaish and Tollin, 1970) and by studying the chemical and physical properties of protein-bound flavin semiquinones.

ESR studies (Guzzo and Tollin, 1964; Ehrenberg et al., 1967) on free flavin semiquinones have indicated unpaired electron spin densities to be greatest at the N(5), N(10), and C(8) positions on the isoalloxazine ring. These results suggest those ring positions as possible sites for electron transfer in flavoenzyme semiquinones.

Unfortunately, the spin density distribution of protein-bound semiquinones has not been measured. Due to the slow rotational motion of the large protein molecule, ESR hyperfine bands cannot be observed. This problem could
be partially solved, however, by ESR studies on oriented crystals of a flavoenzyme in its semiquinone form.

Since the initial work of Massey and Palmer (1966), the semiquinone forms of a number of flavoenzymes have been produced. Hinkson and Bulen (1967) reported the isolation of a flavoprotein from *Azotobacter vinelandii* which has several unusual properties. The semiquinone form is resistant to further reduction, even in the presence of a large excess of dithionite. The semiquinone is also quite resistant to air oxidation. In contrast, the semiquinones of other similar flavoproteins such as *P. elsdenii* flavodoxin (Mayhew and Massey, 1969) are fully reduced by dithionite and react much faster with oxygen. Mayhew, Foust, and Massey (1969) have measured the redox potential of the oxidized/semiquinone flavodoxin system to be -0.115v. No ionization of the "blue" semiquinone to the anionic "red" form was observed up to pH = 10.2, indicating a large shift in the pK of the protein-bound semiquinone.

**Properties of the Fully-reduced Flavins**

The absorption spectrum of fully-reduced (hydroquinone) flavin differs substantially from the spectra of the semiquinone and oxidized forms (Beinert, 1960). The absorption in the visible and near ultraviolet is featureless, with a shoulder at 400 nm and a shoulder at 290 nm on the "tail" of the more intense absorption band at 250 nm (Beinert, 1960).
The ionization of the neutral flavin hydroquinone to the anionic form has a $pK = 6.2$ (Michaelis and Schwarzenbach, 1938; Draper and Ingrahm, 1968). Mayhew et al. (1969) have determined the $pK$ of the protein-bound flavin hydroquinone of $P.\hspace{1em}$ elsdenii flavodoxin to be 5.8. No other $pK$ values for protein-bound hydroquinones have been measured to date. The structures of the neutral and anionic forms are:

$$pK = 6.2$$

The designation of the $N(1)$ position as the site of proton dissociation is based on the following evidence (Ehrenberg and Hemmerich, 1966). Infrared studies indicate the protons at $N(1)$ and at $N(3)$ to be the most acidic. Since alkylation at the $N(3)$ position does not affect the ionization behavior of the flavin hydroquinone, the $N(1)$ position must be the site of ionization.

On the basis of spectroscopic evidence, Dudley et al. (1964) have proposed that the flavin hydroquinone has a bent "butterfly" configuration about $N(10)$ and $N(5)$ which may be in equilibrium, through the coplanar form, with the opposite bent configuration. An absorption shoulder at 400 nm disappears upon protonation ($pH \leq 0$) or ionization.
(pH > 6.2), or upon alkylation of the N(5) position. This absorption has been attributed to the delocalization of the non-bonding electrons at N(5) (Hemmerich et al., 1965). If the flavin molecule were bent about the N(5)-N(10) axis, with N(5) being a quaternary ammonium tetrahedral center in the protonated form, no appreciable delocalization would be expected to occur. The ionization of the N(1) proton would also prevent delocalization of the N(5) electrons to the pyrimidine ring. Alkylation of N(1) and N(10), for steric reasons, would favor the "bent" form. This seems to be the case, as the absorption spectrum of N-1,10-dimethylisoalloxazine shows little absorption in the 400 nm region (Dudley et al., 1964). Replacing the two methyl groups with a -CH₂-CH₂- bridge makes the ring planar, and the expected absorption in the 400 nm region is apparent (Dudley et al., 1964).

Molecular orbital calculations (Fox et al., 1967) on the flavin hydroquinone molecule also indicate a substantial amount of folding. Calculations assuming a planar molecule give very unsatisfactory agreement with experimental data. These calculations indicate that the correct parameterization of N(5) and N(10) is intermediate between tetrahedral and trigonal. Other molecular orbital calculations (Song, 1969) suggest that the methyl perturbation of the spectroscopically active molecular orbitals is less important in the hydroquinone form than in the oxidized flavin ring.
This could be due to the decreased electron withdrawing effect of the ring upon reduction.

Recent x-ray crystallographic data on a series of alkylated flavin analogs have shown that those analogs which have the same spectral properties as the flavin hydroquinone are in a bent configuration (Kierkegaard et al., 1970). The evidence thus seems overwhelmingly in favor of a bent structure for the flavin hydroquinone.

**Spectroscopy of Oxidized Flavins**

An understanding of the spectral transitions in the isoalloxazine ring is important in the study of flavin-protein interactions. Protein-caused perturbations of the electronic transitions would provide information about the environment of the protein-bound flavin.

In most cases, the binding of flavin to a protein results in spectral shifts in the 300 nm-500 nm region of the flavin absorption spectrum (Palmer and Massey, 1966). This region is the most amenable to experimental study, inasmuch as electronic transitions from the aromatic amino acids and from the amide chromophore interfere below 300 nm. A partial resolution of the 450 nm absorption band into three bands is observed in many flavoenzymes (Penzer and Radda, 1967; Palmer and Massey, 1968). Similar spectral shifts and increases in resolution are obtained upon dissolving flavins in non-polar solvents (Harbury et al., 1959; Kotaki et al., 1967). These observations have been
interpreted to indicate a non-polar environment for the protein-bound flavin (Palmer and Massey, 1968).

Due to the intensity of the four major flavin bands \( (\varepsilon > 10^{4} \text{M}^{-1} \text{cm}^{-1}) \), they have been attributed to \( \pi \rightarrow \pi^* \) electronic transitions in the flavin ring. \( n \rightarrow \pi^* \) electronic transitions would also be expected to occur, originating from the nitrogens in the ring system (positions 1, 5, and 10). The shoulder which appears on the long wavelength side of the 450 nm band in the absorption spectrum of riboflavin in a non-polar solvent has been attributed to an \( n \rightarrow \pi^* \) transition (Kotaki et al., 1967). Since the polarization of the flavin fluorescence is constant across this absorption region (thus indicating only a single electronic transition), the resolved bands have alternatively been ascribed to vibronic structure (Weber, 1966). Fluorescence polarization is also constant across the 375 nm band (Weber, 1966), again suggesting that only a single transition occurs in this spectral region. The results of molecular orbital calculations (Fox et al., 1967) on the isoalloxazine ring system conflict with this observation. These calculations predict the presence of an electronic transition at 340 nm, in addition to the transitions at 375 nm and at 450 nm. Circular dichroism and magnetic circular dichroism spectral data (Tollin, 1968a) provide experimental evidence for this third electronic transition. Miles and Urry (1968) also have observed this band in CD studies of FAD, FMN, and
riboflavin and have assigned it as an \( n \rightarrow \pi^* \) transition, based on a correlation of their data with the fluorescence polarization measurements. Another basis for this assignment was the calculation by Song (1969) which predicted an \( n \rightarrow \pi^* \) transition with a singlet energy of 3.34 eV (371 nm). As will be shown in this study, this assignment is probably not correct.

Recent fluorescence polarization measurements (Kurtin and Song, 1968) and circular dichroism studies (Miles and Urry, 1968) have provided evidence for the presence of still another transition (possibly \( n \rightarrow \pi^* \)) at 300 nm. This transition has a low oscillator strength since it is not seen in the flavin absorption spectrum. Molecular orbital theory does not predict a \( \pi \rightarrow \pi^* \) transition in this region (Fox et al., 1967).

### Flavin-protein Interactions

#### Experimental Approaches

The earliest study of flavin-protein interactions began with the resolution of the FMN group from the protein moiety of the "Old Yellow Enzyme" by acidic \((\text{NH}_4)_2\text{SO}_4\) treatment (Åkeson, Ehrenberg, and Theorell, 1963). Recombination of the protein with FMN resulted in a fully active enzyme. The reversible resolution of flavin groups in flavoproteins is most commonly attempted with acidic \((\text{NH}_4)_2\text{SO}_4\) (Warburg and Christian, 1938), trichloroacetic
acid precipitation (Hinkson, 1968) or dialysis against KBr (Mayhew and Massey, 1969). That such treatments are effective illustrates that, in most cases, the flavin group is not covalently bound to the protein. The only known example of a covalent bond between protein and flavin is found in the enzyme succinic dehydrogenase (Singer and Kearney, 1963).

The reversible resolution of flavoenzymes has made possible a more complete study of the flavin-protein interactions which may govern the specificity of the redox active flavin group in catalysis. In most flavoenzymes, the bound flavin is no longer fluorescent (Palmer and Massey, 1968). Thus, the measurement of flavin binding by fluorescence quenching techniques (Theorell and Nygaard, 1954) provides a very sensitive method to study the kinetics and binding energies for the flavin coenzymes.

To date, the investigation of flavin-protein interactions has been approached by two methods. The first is to study the protein binding of flavin analogs and, if bound, to measure the enzymatic activity of the reconstituted protein-flavin analog complex. The other approach is the study of flavin binding to a chemically-modified apoprotein. The chemical modification of an amino acid residue suspected of interacting with the flavin chromophore has implicated lysyl groups and tyrosyl group(s) in the Old Yellow Enzyme (Åkeson et al., 1963).
The quenching of flavin fluorescence by the protein moiety has led to several hypotheses of flavin-protein interaction. From the observations that N(3) substituted flavins were non-fluorescent (Kuhn and Boulanger, 1936) and that tyrosyl iodination inhibited flavin binding to the Old Yellow Enzyme, Theorell (1959) suggested a possible protein-flavin linkage to be a hydrogen bond between the N(3) flavin position and the phenolic hydrogen of a tyrosyl residue. This hypothesis has been disproven by the observation that N(3) substituted flavins actually are nearly as fluorescent as unsubstituted flavins (Harbury and Foley, 1958; Chassey and McCormick, 1965a). Also, 3-methylFMN is about 69% as effective as FMN in restoring enzymatic activity to the apoenzyme of the Old Yellow Enzyme (Tsibris, McCormick, and Wright, 1966).

**Protein Complexes of Isoalloxazine Ring-modified Flavins**

Investigations of the importance of the flavin N(3) position in several flavoenzymes have been reported. Tsibris *et al.* (1966) restored partial catalytic activity when 3-methylFMN was added to the apoenzymes of several FMN containing flavoenzymes: reduced nicotinamide adenine dinucleotide phosphate (NADPH) dehydrogenase (Old Yellow Enzyme), yeast NADPH-cytochrome c reductase, NADH oxidase (isolated from a camphor-degrading pseudomonad), and rabbit liver pyridoxamine phosphate oxidase. Strong binding of
the 3-methylFMN to the NADH apooxidase was indicated by its competitive inhibition of restoration of activity by FMN. A flavoprotein isolated from egg white binds 3-methylriboflavin with one-sixth the affinity of riboflavin (Rhodes et al., 1959).

Visser, McCormick, and Veeger (1968) have investigated the binding and catalytic activity of N(3) substituted FAD with apolipoamide dehydrogenase. The unbound 3-methyl-FAD is quite similar to FAD with respect to fluorescence and absorption properties (Chassey and McCormick, 1965a). Binding of either 3-methylFAD or 3-carboxymethylFAD resulted in fairly active complexes towards the artificial substrate 2, 6-dichlorophenol indophenol. On the other hand, the 3-methylFAD analog was only 15% as effective as FAD in restoring lipoate dehydrogenase activity while the 3-carboxymethylFAD analog was inactive. The association constants for the 3-methylFAD and the 3-carboxymethylFAD were, respectively, one-third and one-tenth that of FAD. The above results indicate that protein interaction with the flavin N(3) position probably occurs but is not crucially important in binding or in catalytic activity.

One example of an absolute requirement for an unsubstituted N(3) position to restore catalytic activity has been observed in apo-D-amino acid oxidase (Chassey and McCormick, 1965b). Competitive inhibition with FAD is also quite low, thereby indicating a decreased association constant.
The effect of modifications of other sites on the flavin ring on protein-binding and restoration of catalytic activity have not been investigated systematically. Tsibris et al. (1966) have shown that replacement of the methyl groups with chlorine at positions 6 and 7 reduced the restored catalytic activity of the apoenzymes discussed above through changes in oxidation-reduction potential of the flavin, since, in general, this analog was bound fairly strongly. Modification of the 2-carbonyl group in FAD (as the 2-morpholinoFAD analog) resulted in complete inactivity in restoring enzymic properties to apolipoamide dehydrogenase (Visser et al., 1968). The replacement of the six and seven methyl groups with ethyl groups had no inhibitory effect in restoring the activity of apo NADPH-cytochrome c reductase (Arsenis and McCormick, 1964).

Protein Complexes of Side-chain Modified Flavins

Most studies on side-chain modified flavin interactions with an apoprotein have been concerned with the 5' group on the ribityl chain. Three readily available flavins with different 5' groups are FMN, riboflavin, and FAD. Riboflavin and FAD can restore activity to the apoenzyme of the FMN-containing Old Yellow Enzyme; however, their association constants are much lower than that of the FMN protein complex (Akeson et al., 1963). Fluorescence quenching is also observed upon FAD and riboflavin binding, as is the case with FMN binding (Nygaard and Theorell, 1955).
Removal of the ribityl side-chain hydroxyl groups of FMN, to form the 5'-hydroxypentylFMN, restored 56% of the native activity (Tsibris et al., 1966). No measurement of fluorescence quenching of the 5'-hydroxypentylFMN by the apoenzyme was made.

Binding of 5'-hydroxypentylFMN produced a more active product than FMN binding with apo NADPH-cytochrome c reductase; however, this analog restored very little activity with the apoenzyme of NADH oxidase (Tsibris et al., 1966). Arsenis and McCormick (1964) investigated the effect of FMN side-chain modification on generating activity with apo NADPH-cytochrome c reductase in more detail. They found that the activities of flavin phosphates having different side chains progressively decreased as the chain was shortened (i.e., D-ribityl > D-erythrityl > D,L glyceryl). The reported restoration of activity by deoxyFMN relative to FMN (62%) in this work is at variance with a later report that FMN gives only 52% of the activity obtained with deoxyFMN (Tsibris et al., 1966). No explanation was given for this discrepancy. Arsenis and McCormick (1964) also found approximately the same activities for the 2'-deoxyriboflavin 5'-phosphate and the D-araboflavin 5'-phosphate analogs as for deoxyFMN, using apo NADPH-cytochrome c reductase. This is indicative of the importance of the 2' hydroxyl group and of a D-configuration of that hydroxyl group in the restoration of catalytic activity.
The 2' side-chain hydroxyl group is also important in restoring the catalytic activity of the apoenzyme of the FAD-containing D-amino acid oxidase (Chassey and McCormick, 1965b). Thus, the 5'-deoxyFAD and the 2'-deoxyFAD analogs restored only 20% of the activity obtained with FAD. FAD analogs with shorter side chains (3 and 4 carbons), with and without hydroxyl groups, competitively inhibited the restoration of activity by FAD. This is suggestive that these analogs were tightly bound to the protein but were catalytically inactive. No data were given on the inhibitory activity of 5'-deoxyFAD and 2'-deoxyFAD.

The importance of the nature of the flavin side chain is also apparent in the binding studies of a number of flavin analogs to apo egg white flavoprotein (Rhodes et al., 1959). This flavoprotein has riboflavin as its naturally occurring flavin group but has no known catalytic activity. FMN and FAD were qualitatively less tightly bound than riboflavin. Riboflavin was bound with twenty times the affinity of lumiflavin and 9-formylmethylisoalloxazine.

These studies on several unrelated flavoproteins illustrate the importance of the side chain in binding and in catalytic activity. The nature of the groups at the 5' position and the chain length are expectedly important. Of more interest is the importance of the D-configuration of the 2' hydroxyl group in the restoration of catalytic activity.
Possible Protein Groups Important in Flavin-protein Interactions

Protein-bound flavin differs in spectral properties from free flavin in several respects (Palmer and Massey, 1968): (1) the flavin fluorescence is quenched in the majority of flavoenzymes; (2) a small hypochromism of the 450 nm absorption band is usually observed; and (3) the 450 nm band is sometimes resolved and a "tail" on the long wavelength side is normally observed.

As mentioned previously, complexation of the flavin and adenine aromatic rings produces some of these effects (fluorescence quenching, hypochromism and long wavelength tailing). Other aromatic compounds also form non-covalent complexes with flavin in aqueous solution quite readily (Penzer and Radda, 1967), most significantly the aromatic groups found in protein (tyrosyl and tryptophyl) (Tollin, 1968b). The spectral properties of these complexes are similar to FAD. Resolution of the 450 nm band occurs with flavins dissolved in non-polar solvents. Little or no hypochromism and no long wavelength tailing are observed under these conditions (Kotaki et al., 1967). The hypochromism and "tailing" of the 450 nm absorption band seem to be produced only by proximity of an aromatic ring to the flavin ring.

With the flavinyl amides of tryptophan, tyrosine, and phenalanine (Föry, MacKenzie, and McCormick, 1968;
MacKenzie, Föry, and McCormick, 1969), both fluorescence quenching and hypochromism is evident. As indicated previously, NMR studies (Föry et al., 1970) suggest a stacked, planar configuration. These studies also indicate that the primary interaction of the amino acid side chain is with the benzenoid portion of the flavin ring.

The amino acids tyrosine and tryptophan have long been suspected of being important in protein-flavin interactions (Penzer and Radda, 1967). Iodination of the tyrosines of flavin-free Old Yellow Enzyme (Åkeson et al., 1963) and of one tyrosine in apo NADPH-cytochrome c reductase (Strittmatter, 1961) completely blocks FMN binding. More recently, Hinkson (1968) found that protein-bound FMN protected two tyrosines from nitration in the Shethna flavoprotein. Nitration of four of the five tyrosines eliminated 90% of the FMN binding capacity.

No definitive evidence has been reported for tryptophan-flavin interaction in flavoenzymes. McCormick (1970) has noted the release of FMN in C. pasteurianum flavodoxin upon N-bromosuccinimide oxidation of two of the four tryptophans in the protein. It should also be pointed out that treatment of flavodoxin with sodium mersalyl releases the FMN, thus implicating thiol participation in the binding (Knight and Hardy, 1967). Since thiol groups can be readily oxidized by N-bromosuccinimide (Peters, 1959), the above-cited evidence for a tryptophan-flavin interaction in flavodoxin is by no means unequivocal.
The Relation of Redox Activity to Flavoenzyme Properties

Although flavoenzymes are generally quite specific as regards the chemical nature of the electron donating substrate, they are reactive with a wide variety of electron accepting compounds. The relative reactivity with these electron acceptors can vary substantially and has served as a basis to classify a number of flavoenzymes (Massey et al., 1969a). Flavoproteins are classed as oxidases on the basis of the high reactivity of their hydroquinone forms with molecular oxygen to form the fully-oxidized flavin. In contrast, their fully-reduced forms have very low reactivity with one electron acceptors such as ferricyanide or cytochrome c. (For a listing of the relative reactivities, see Massey et al., 1969a.) No data are available on the reactivity of the semiquinone forms of the oxidases.

Another group of flavoenzymes are classed as dehydrogenases due to the high reactivity of their semiquinone forms with one electron acceptors and relatively low reactivity with oxygen. The hydroquinone forms of the dehydrogenases are also quite reactive with oxygen, generally yielding the semiquinone, although only a few measurements have been made.

Massey et al. (1969a) have correlated a number of properties of the flavoprotein oxidases and of the flavoprotein dehydrogenases. In general, the oxidases form
anionic (red) semiquinone forms upon reduction either with dithionite or by illumination in the presence of EDTA, while the dehydrogenases form the neutral (blue) semiquinones. Using flash photolysis techniques, Vaish and Tollin (1970) have observed that the neutral flavin semiquinone reacts at least $10^5$ times more slowly with oxygen than the anion radical. Thus, this difference may provide at least part of the explanation for the oxygen stability of the dehydrogenase semiquinones. The protein-bound flavin (in the oxidized form) of the oxidases forms sulfite adducts quite readily while the dehydrogenases do not. Studies with model flavin compounds (Müller and Massey, 1969) implicate the N(5) position of the isoalloxazine ring as the site of sulfite addition.

Massey et al. (1969a) have interpreted the correlation between sulfite addition and formation of an anionic semiquinone to be due to the presence of a positively charged protein group near the 4 position of the flavin. The positively charged group would be responsible for the strong binding of benzoate and other carboxylic acids to D-amino acid oxidase (Massey and Ganther, 1965). This group has a pK = 8.2, as judged by the pH effect on sulfite addition to D-amino acid oxidase (Massey et al., 1969a). Conversely, it was postulated that an anionic group on the dehydrogenases stabilizes the positively charged N(5) position of the neutral semiquinone and electrostatically repels the negatively
charged sulfite ion (Massey et al., 1969a). This difference in charge in the vicinity of the flavin hydroquinone could be a factor in determining the mechanism of oxygen oxidation. The oxidation of a fully-reduced oxidase is a two-electron process with hydrogen peroxide as a product, while the oxidation of a dehydrogenase flavin hydroquinone would result in a one-electron transfer to oxygen to form the superoxide ion and the flavosemiquinone (Fig. 2). Further evidence in support of this proposal is the observation that the fully-reduced dehydrogenase flavoproteins produce significant amounts of superoxide ion upon air oxidation while the fully-reduced flavoprotein oxidases do not (Massey et al., 1969b).

Very little work has been done on the reactivity of flavin semiquinones with oxygen or other electron acceptors. Shethna, Wilson, and Beinert (1965) and Hinkson and Bulen (1967) have shown that the flavoprotein isolated from Azotobacter vinelandii has a "blue" semiquinone form which is quite stable to air oxidation and to reduction by dithionite but is oxidized rapidly by cytochrome c and by ferricyanide. Since urea treatment increases the rate of semiquinone oxidation by O₂ and eliminates the resistance to dithionite reduction, Hinkson and Bulen (1967) have ascribed these above-mentioned semiquinone properties to protein-flavin interactions. The semiquinone form of other dehydrogenase flavoenzymes (e.g., P. elsdenii flavodoxin;
Fig. 2. Postulated Mechanism for Oxygen Oxidation of Hydroquinone Form of Oxidases and Dehydrogenases (Massey et al., 1969a).
Mayhew and Massey, 1969) are more readily oxidized by oxygen (although still slowly) and are reduced by dithionite.

Summary

Although some progress has been made in the study of protein-flavin interactions, there are still many problems that require further elucidation. Particularly lacking have been investigations of the physical and chemical properties of flavin analog-protein complexes which would provide information concerning:

1. Protein quenching of flavin fluorescence
2. Sites on the flavin molecule important in protein binding
3. The participation of protein groups in flavin binding and in modifying the properties of the several flavin redox forms

The present study represents an effort in this direction. In addition, the relations between flavin redox form and protein conformation, the spectroscopy of the protein-bound flavin chromophore and correlations between enzyme redox properties and flavin environment for a number of flavoenzymes are examined.

The Shethna flavoprotein from *Azotobacter vinelandii* was chosen as a particularly suitable model system for this type of study. This is because of its stability (with and
without the flavin group) towards denaturation, to its ease of isolation, and to the stability of its semiquinone form to air oxidation. However, comparisons between the properties of this protein and other flavoenzymes are made whenever possible.
EXPERIMENTAL

Materials

Flavoenzymes

The Shethna flavoprotein was isolated as outlined below from cells of *Azotobacter vinelandii* using the method of Hinkson and Bulen (1967). *Peptostreptococcus elsdenii* and *Clostridium pasteurianum* flavodoxins were gifts from Dr. V. Massey, Department of Biological Chemistry, the University of Michigan, Ann Arbor, Michigan. L-amino acid oxidase was isolated from the venoms of *Crotalus adamanteus* and of *Crotalus atrox* by the method of Wellner and Meister (1960). The dried venoms were obtained from Sigma Chemical Company, St. Louis, Missouri. *Rhodospirillum rubrum* flavodoxin was a gift from Dr. M. Cusanovich of this Department. Thin-layer chromatography identified the flavin group as FMN. Glucose oxidase from *Aspergillus niger* was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. It was further purified by column chromatography and ammonium sulfate precipitation, following the final stages of the purification procedure of Swoboda and Massey (1965). Ferredoxin-TPNH reductase was isolated from fresh spinach leaves, obtained at a local market, using the method of Shin, Tagawa, and Arnon (1963). The concentrations of
flavoenzymes were determined spectrophotometrically using the published extinction coefficients given in Table II.

**Flavin Analogos**

Flavin adenine dinucleotide (FAD) was obtained from Sigma Chemical Company, St. Louis, Missouri. Riboflavin, isoriboflavin, and flavin mononucleotide (FMN) were obtained from Calbiochem, Los Angeles, California. N-10-ω-hydroxy-pentylisoalloxazine (deoxyriboflavin), N-10-ω-carboxybutylisoalloxazine, and 3-methylriboflavin were gifts from Dr. D. McCormick, Division of Biological Sciences, Cornell University, Ithaca, New York. IsoFMN and tetra-O-acetyl riboflavin were gifts from Dr. V. Massey, Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan. The 3-methylFMN and 2-thioFMN were gifts from Dr. P. Hemmerich, University of Konstanz, Konstanz, Germany. DeoxyFMN was synthesized by phosphorylating N-10-ω-hydroxy-pentylisoalloxazine according to the method of Flexser and Farkas (1952) and purified by DEAE-cellulose column chromatography. Lumiflavin was previously synthesized in our laboratory using the method of Guzzo and Tollin (1963). All flavin derivatives were homogeneous upon thin-layer chromatography on cellulose plates, using either 5% K$_2$HPO$_4$ or butanol, acetic acid, water (4:1:5 v/v/v) as the developing solvent. Because of the unexpected results obtained with this derivative (see below), its identity was confirmed using thin-layer chromatography and infrared spectroscopy. 
<table>
<thead>
<tr>
<th>Flavoenzyme</th>
<th>( \varepsilon_M )</th>
<th>( \lambda_{\text{nm}} )</th>
<th>Flavin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shethna</td>
<td>10,600</td>
<td>452</td>
<td>FMN</td>
<td>Hinkson and Bulen (1967)</td>
</tr>
<tr>
<td>Flavoprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. elsdenii</td>
<td>10,200</td>
<td>445</td>
<td>FMN</td>
<td>Mayhew and Massey (1969)</td>
</tr>
<tr>
<td>Flavodoxin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. pasteurianum</td>
<td>9,100</td>
<td>443</td>
<td>FMN</td>
<td>Knight and Hardy (1966)</td>
</tr>
<tr>
<td>Flavodoxin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. rubrum</td>
<td>10,500(^a)</td>
<td>460</td>
<td>FMN</td>
<td>Shin et al. (1963)</td>
</tr>
<tr>
<td>Flavodoxin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferredoxin-TPNH Reductase</td>
<td>10,300</td>
<td>458</td>
<td>FAD</td>
<td>Swoboda and Massey (1964)</td>
</tr>
<tr>
<td>Glucose Oxidase</td>
<td>14,100</td>
<td>450</td>
<td>FAD</td>
<td></td>
</tr>
<tr>
<td>L-amino Acid Oxidase</td>
<td>11,300</td>
<td>450</td>
<td>FAD</td>
<td>Massey and Curti (1966)</td>
</tr>
</tbody>
</table>

\(^a\) Assumed value
solvent. Most flavin concentrations were determined spectrophotometrically at 445 nm using a molar extinction of 12,200 liters-mole\(^{-1}\)cm\(^{-1}\). The flavins having different extinctions and absorption maxima are listed in Table III.

**TABLE III**

Spectral Properties of Flavin Analogs

<table>
<thead>
<tr>
<th>Flavin</th>
<th>(\varepsilon_{\text{Molar}})</th>
<th>(\alpha_{\text{mm}})</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD</td>
<td>11,300</td>
<td>450</td>
<td>Penzer and Radda (1967)</td>
</tr>
<tr>
<td>IsoFMN</td>
<td>6,700</td>
<td>448</td>
<td>Berezovski and Radinova (1958)</td>
</tr>
<tr>
<td>Isoriboflavin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-thio-FMN</td>
<td>12,200</td>
<td>485</td>
<td>Dudley et al. (1964)</td>
</tr>
</tbody>
</table>

**Other Materials**

Crystalline bovine serum albumin was obtained from Pentex, Inc., Kankakee, Illinois. Sephadex G-100 and G-10 were from Pharmacia Fine Chemicals, Inc., New Market, New Jersey. DEAE-cellulose, Biogel P-2 and P-10 were purchased from Calbiochem, Los Angeles, California. Sucrose (special enzyme grade) was obtained from Mann Research Laboratories, Inc., New York, New York. Sodium dithionite (practical grade) was from Eastman Organic Chemicals, Rochester, New York. NADH was obtained from the Sigma Chemical Company, St. Louis, Missouri. Methyl viologen was obtained from K and K Laboratories, Inc., Hollywood, California. All other chemicals were of reagent grade from commercially available sources.
Ion-low water was prepared by passing distilled water through a Barnstead mixed-bed ion-exchange column. This water was used in the preparation of all solutions. Culture media were also prepared using distilled water.

**Instruments**

Routine absorption measurements were made at fixed wavelengths with the Gilford Model 240 UV-VIS spectrophotometer. Routine absorption spectra were obtained with a Coleman Model 124 Hitachi double-beam spectrophotometer, equipped with a Beckman Model 1005 10-inch linear-log recorder. The Cary Model 14R spectrophotometer, fitted with a 0-1.0 absorbance unit slide wire, was used for obtaining more accurate absorption spectra. This spectrophotometer, fitted with a 0-0.1 absorbance unit slide wire, was also used for difference spectra measurements. The Cary Model 60 spectropolarimeter, equipped with the Model 6001 circular dichroism attachment, was used in measuring circular dichroism (CD) spectra. Curve resolution of absorption and CD spectra using Gaussian functions was accomplished using the DuPont Model 310 curve resolver.

Cylindrical and rectangular quartz cells used in spectrophotometric measurements were obtained from Precision Cells, Inc., Hicksville, New York. Thunberg quartz cuvettes of 10 mm pathlength for anaerobic spectral measurements were purchased from Precision Cells, Inc. and also from Helma Cells, Inc., Jamaica, New York.
Bacterial cells were broken with a French pressure cell which was driven by an electrically-operated hydraulic press. This instrument was obtained from the American Instrument Company, Silver Springs, Maryland. The bacterial cells were harvested using a Sharples Super Speed centrifuge, type T-1, at approximately 25,000 rpm at room temperature. Low-speed centrifugations were carried out with a Sorvall refrigerated Model C2-B centrifuge utilizing the Model SS-34 rotor. High-speed centrifugations were done using the Spinco Model L ultracentrifuge with either the type 30 or the Ti-50 rotor. Experiments utilizing the analytical ultracentrifuge were performed on a Spinco Model E ultracentrifuge equipped with schlierien optics. The Yphantis cell, the double sector cell and the double sector synthetic boundary cell were used, when applicable, in the measurements. The photographic plates of the refractive index patterns were analyzed on a Nikon microcomparator.

Fluorescence quenching was measured using a fluorimeter built by Dr. G. Tollin. This instrument utilized band pass primary and secondary filters. The excitation beam was mechanically chopped and the emission signal was detected utilizing a lock-in amplifier. Ten mm pyrex rectangular cells were used in all experiments. Signals were recorded on a Sanborn Model 151 recorder.

Electron spin resonance measurements were made at room temperature with a Varian V-4501 electron paramagnetic...
resonance spectrometer equipped with a Fieldial control utilizing Varian aqueous solution cells.

pH measurements were made using a Leeds and Northrup pH meter equipped with the Leeds and Northrup miniature pH electrode assembly. Nitrogen gas was purified from contaminating oxygen by passing it over hot copper filings in a furnace obtained from E. F. Sargent Company, Chicago, Illinois. A duo-seal oil vacuum pump (Welch Scientific Company, Skokie, Illinois) was used for degassing samples.

Experimental Procedures

Maintenance and Growth of Azotobacter vinelandii Cultures

The cultures used in the initial phase of this work were obtained from Dr. W. A. Bulen, Kettering Research Laboratories, Yellow Springs, Ohio, and from the United States Department of Agriculture Type Collection, Peoria, Illinois. The culture used in the majority of the project was obtained from the American Type Culture Collection, Washington, D. C.

Cultures of Azotobacter vinelandii, strain 0, were maintained on agar slants at 0°C. Fresh agar slants were inoculated every month. The agar gel was prepared by dissolving agar (2% w/v) in nitrogen-free Burk's media (Table IV) containing 2% sucrose.

For growing batch quantities of bacterial cells, 150 ml of media in a 500 ml Erlenmeyer flask was inoculated
<table>
<thead>
<tr>
<th>Salt</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>2.0</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.8</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.2</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>0.2</td>
</tr>
<tr>
<td>CaSO$_4$</td>
<td>0.05</td>
</tr>
<tr>
<td>Fe solution$^a$</td>
<td>1 ml/liter</td>
</tr>
<tr>
<td>Mo solution$^b$</td>
<td>1 ml/liter</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20</td>
</tr>
</tbody>
</table>

$^a$Fe solution 1.5g·FeSO$_4$·5H$_2$O + 1.14g·citric acid in 100 ml H$_2$O

$^b$Mo solution 0.2525g·Na$_2$MoO$_4$·2H$_2$O in 100 ml H$_2$O
and agitated in a shaker bath at 30°C. Normally, liquid cultures were maintained in this manner, with cultures being changed every two days by inoculating a flask of fresh medium with ≈10 ml of the old culture. A 12-liter glass carboy containing 8 liters of medium was then inoculated with one 150 ml culture. Four carboys of inoculated medium were placed in a large metal tank of water maintained at 30°C. The cultures were grown for 20-24 hours (the late, linear part of the growth phase) under forced aeration at a pressure of 6 p.s.i. The cells were harvested by centrifugation and stored at -10°C without washing.

Isolation of the Shethna Flavoprotein

This procedure follows that of Hinkson and Bulen (1967). Wet cell paste (22g.) was suspended in 60 ml of cold 0.025M phosphate buffer, pH = 7.0. The cells were broken by passing through a French pressure cell at 16,000 p.s.i. Unbroken cells and large cell debris were removed by centrifugation of the broken cell suspension for 10 min. at 12,000 xg. The supernatant was then centrifuged for 120 min. at 105,000 xg, followed by treatment with 5 ml of a 2% protamine sulfate solution, pH = 6.0, per 25 ml of supernatant to precipitate nucleic acid material. After standing for 30 min. at 0°C, the suspension was centrifuged at 12,000 xg for 15 min.

The supernatant was then applied to a DEAE-cellulose column equilibrated with 0.025M phosphate, pH = 7.0. A
1.6 x 20 cm. column was used for 500 ml of supernatant. After washing the column with 0.025M phosphate, pH = 7.0 and 0.15M NaCl-0.025M phosphate, pH = 7.0, in that order, to remove contaminating material, the flavoprotein (a greenish-yellow band) was eluted with 0.4M NaCl-0.025M phosphate, pH = 7.0. Solid (NH₄)₂SO₄ was added to the flavoprotein fraction to 75% of saturation. After standing for 30 min., precipitated protein was removed by centrifugation at 15,000 xg for 15 min. The yellowish-green supernatant (the Shethna flavoprotein) was then either dialyzed to remove the (NH₄)₂SO₄ or crystallized.

Crystallization was accomplished by wrapping a dialysis bag, containing the flavoprotein in the 75% (NH₄)₂SO₄ solution, in a paper towel and placing in the refrigerator. After 3-4 days, small crystals as well as some precipitated amorphous material appeared and were centrifuged. This technique was normally used to prepare very concentrated solutions of flavoprotein. The flavoprotein exhibited the same properties and purity whether or not the crystallization process was used.

From the data of Hinkson and Bulen (1967), a concentration of 1 mg/ml of flavoprotein had an absorbance at 358 nm of 0.282. The molar extinction of the oxidized form at 452 nm was 10,600 M⁻¹cm⁻¹. The molar extinction at 358 nm for both the oxidized and semiquinone forms (the isosbestic point) was 8,820 M⁻¹cm⁻¹. The molar extinction of the semiquinone form at 580 nm was 5,440 M⁻¹cm⁻¹.
Preparation of the Apoprotein

The procedure used for resolving the flavin from the protein is a modification of that described by Hinkson (1968). Dithiothreitol was added to an aqueous solution of the flavoprotein (≈1 mg/ml) to a final concentration of 1 x 10^{-3}M. At 0°C and under dark conditions, a 30% (w/v) aqueous solution of trichloroacetic acid was added to the protein solution to a final concentration of 3%. The precipitated protein was collected by centrifugation at 12,000 xg for 15 min. After decantation, the pellet was washed with 3% trichloroacetic acid and recentrifuged. Normally after one washing, the protein pellet was white in color. If any yellow color still remained, the precipitate was washed again. The precipitated apoprotein was dissolved in 1-2 ml of 0.1M Tris buffer, pH = 8.5. If any precipitated protein would not solubilize, it was removed by centrifugation. At pH = 8.5, the apoprotein was stable for several weeks. The apoprotein was stable for only several days at pH = 7.0. A protein concentration of 1 mg/ml had an absorbance at 280 nm of 1.270 as determined by the Lowry et al. (1951) method of protein determination (Fig. 3). If the precautions of adding dithiothreitol and dark conditions were not observed, a large molecular weight protein species was observed upon column chromatography on Sephadex G-100. Similarly, two electrophoretic bands were observed upon cellulose acetate electrophoresis. This high molecular
Fig. 3. Absorption at 280 nm as a Function of Shethna Apoprotein Concentration.

The protein solutions were dissolved in 0.025M phosphate buffer, pH = 7.0. Protein concentrations were estimated by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.
weight material had a different ultraviolet absorption and circular dichroism spectra than the apoprotein and would not rebind FMN. No systematic investigation into the structure of this material was made. Since flavin is a good oxidizing agent under acid conditions, it is presumed that thiol oxidation to form intermolecular disulfide bonds could occur and that photooxidation of tyrosine and/or tryptophan might also occur.

The technique of dialyzing the holoprotein in 2M KBr, as was used in resolving the FMN group from *P. elsdenii* flavodoxin (Mayhew and Massey, 1969), was not successful in dissociating the FMN-protein complex of the Shethna flavoprotein.

**Circular Dichroism Measurements**

All circular dichroism measurements were taken at ambient temperatures. Ten mm. cylindrical quartz cells were normally used in the spectral region from 250-600 nm. Measurements in the 190-250 nm regions were accomplished using 1 mm. cylindrical quartz cells. CD measurements on the half-reduced flavoenzymes from 700 nm-250 nm were made using 10 mm., rectangular quartz Thunberg cuvettes. The cuvettes were positioned in the instrument by utilizing a cell holder from the Cary 14 spectrophotometer. Where solubility was limited, as in the case of some of the flavin derivatives, 25 mm. and 50 mm. quartz cylindrical cells were used.
During all measurements, a time constant and scan speed were chosen so as to provide a good signal-to-noise ratio. The programmed slit width system was used in the 190-600 nm spectral region. A constant slit width of 0.5 mm. was used in recording spectra in the 600-700 nm spectral range. To avoid absorption artifacts, the dynode voltage across the photomultiplier tube never exceeded 0.45 kilovolts.

The output of the Cary instrument is in terms of ellipticity, measured in degrees and expressed as \( \Theta \). To convert to molecular ellipticity \( (\Theta) \), the following equation was used:

\[
(\Theta) = \frac{\Theta}{l \times c}
\]

where \( l \) is the cell pathlength in cm. and \( c \) is the concentration of the sample in decimoles-cm\(^{-3}\). The molecular ellipticity thus has the units degrees-cm\(^2\)-decimole\(^{-1}\). Since the amide bond is the major chromophore below 250 nm, measurements in the far ultraviolet region were expressed in terms of mean residue ellipticity using the relation:

\[
(\Theta)_m = \frac{\Theta \times 130}{l \times c}
\]

where 130 is the mean residue weight and \( c \) is the protein concentration in mg-ml\(^{-1}\). Molecular ellipticity is related to the difference in molar extinction of left and right circularly polarized light \( (\epsilon_L - \epsilon_R) \) by the relation:

\[
(\Theta) = 3300 (\epsilon_L - \epsilon_R).
\]
Analytical Ultracentrifugation Experiments

The sedimentation coefficient of the Shethna flavoprotein was measured on four samples simultaneously using two double sector cells (Adams and Adams, 1970). To separate the sedimenting refractive index gradients on the photographic plate, a positive and a negative wedge window were used on the two cells. A rotor speed of 59,767 rpm at a temperature of 20°C was used in the experiment. Pictures were taken every 16 min. after the rotor speed was attained. The plates were measured and the data plotted as ln r vs. t (where r is the distance sedimented and t is expressed in minutes). The sedimentation coefficient was then estimated from the slope of the linear plot and corrected to the standard conditions of a water solvent at 20°C.

The diffusion coefficient was measured utilizing a double sector synthetic boundary cell. The rotor speed in these experiments was 10,000 rpm and the temperature was 20°C. The photographed refractive index gradients were traced on graph paper. The area under the spreading peak was measured with a planimeter. The diffusion coefficient was then estimated from the slope of the linear plot of \( A^2/H^2 \) vs. t, where A is the area under the peak and H is the height of the peak; t is expressed in seconds.

The molecular weight of the flavoprotein was calculated from the sedimentation and diffusion coefficients at infinite dilution using the Svedberg equation:
M = \frac{RT S_{20,w}}{D_{20,w} (1-\bar{\nu}_p)}

where $S_{20,w}$ is the sedimentation coefficient at infinite dilution, $D_{20,w}$ is the diffusion coefficient at infinite dilution, $R = \text{gas constant (8.314 joules-deg}^{-1}\text{mole}^{-1})$, $T$ is the absolute temperature, $\bar{\nu}$ is the partial specific volume of the protein and $\rho$ is the density of the solvent. The procedures for these determinations are outlined by Svedberg and Pedersen (1940). The partial specific volume of the protein was calculated from the amino acid composition by the procedure of Cohen and Edsall (1943).

The molecular weight of the Shethna flavoprotein was also determined by the short-column sedimentation equilibrium technique of Yphantis (1964). The rotor speed was 20,000 rpm and the temperature was 20°C. Equilibrium was established after 20 hours. The slope of the linear plot of $\ln y/r$ vs $r^2$ (where $y$ is the height of the refractive index gradient from the solvent baseline and $r$ is the distance from the center of rotation) was used to calculate the molecular weight using the relation:

$$M = \frac{2RT(slope)}{W^2(1-\bar{\nu}_p)}$$

$W$ is the angular acceleration in radians/sec. All other symbols are defined above.
Amino Acid Composition

Amino acid analysis on dried, salt-free samples of the flavoprotein and of the apoprotein were performed by the AAA Laboratory, Seattle, Washington. Cysteine was estimated as cysteic acid after performic acid oxidation (Hirs, Moore, and Stein, 1956) and as carboxymethylcysteine with and without prior reduction by mercaptoethanol. Tryptophan was determined after Ba(OH)$_2$ hydrolysis (Dreize and Reith, 1956). Tryptophan was also determined in our laboratory spectrophotometrically (Goodwin and Morton, 1946) and by N-bromosuccinimide oxidation (Peters, 1959). Both of these determinations were made on the apoprotein as the flavin chromophore interferes with the analysis.

Tyrosine Spectrophotometric Titration

The tyrosyl ionization behavior of the apoprotein and of the holoprotein was followed spectrophotometrically at 245 nm. Absorbance measurements were made directly after adding microliter amounts of 5N NaOH to the protein solution in 0.1M glycine buffer. pH measurements were made in the spectrophotometer cell after the absorbance measurement.

The tyrosyl ionization of the semiquinone form was done anaerobically on individual samples. A calculated amount of 5N NaOH was added to the sidearm of a quartz Thunberg cuvette. After thoroughly flushing out any oxygen
by alternate evacuating with an oil pump and purging with oxygen-free nitrogen, the base was tipped into the protein solution and the change in absorbance at 245 nm immediately determined. The change at 245 nm was immediately determined. The change at 580 nm was quite small during this procedure indicating negligible loss of semiquinone. The pH of the solution was then determined after opening the cuvette.

To calculate the extent of ionization, the change in molar extinction at 245 nm was assumed to be 11,500M⁻¹cm⁻¹ (Wetlaufer, 1962). The tyrosyl ionization of the semiquinone form had to be followed anaerobically and quickly due to the increased rate of oxidation by O₂ and to disproportionation of the radical at high pH. For this latter reason the tyrosyl ionization measurements represent initial values for all three forms. No measurements were made on the total number of tyrosines titrated upon long standing at a certain pH. Reverse titrations on the apoprotein and holoprotein indicated reversibility of ionization. A reverse titration could not be performed on the semiquinone form because of the previously-mentioned technical difficulties.

Determination of Binding Constants with a Number of Flavin Analogs

The association constants of all flavin analogs with the apoprotein were determined by fluorescence quenching experiments in 0.025M phosphate, pH = 7.0 at room temperature. A microliter quantity of apoprotein was added to 2 ml of
flavin solution and the residual flavin fluorescence at 530 nm was measured at equilibrium. The micropipet was calibrated spectrophotometrically by pipetting the protein solution into 2 ml of buffer and measuring the absorbance at 280 nm. Flavin concentrations were measured spectrophotometrically at 445 nm. For most of the flavins, the fluorescence was assumed to result from only unbound flavin. In the case of deoxyriboflavin, N-10-ω-carboxybutylisalloxazine and FAD, the residual fluorescence upon adding a 5-fold molar excess of apoprotein was assumed to result from the protein-flavin complex. The quenching data were then corrected for this residual fluorescence.

To calculate the association constant, a simple 1 : 1 equilibrium was assumed: $P + F \rightleftharpoons PF$. The association constant $K_a$ can then be expressed as:

$$K_a = \frac{(PF)}{(P)(F)}$$

The fraction of protein-flavin complex to total protein ($d$) is given as:

$$d = \frac{(PF)}{(P_{Total})}$$

The total protein concentration ($P_{Total}$) is equal to free protein plus bound protein:

$$(P_{Total}) = (P) + (PF)$$

Solving for $(PF)$ and $(P)$ from equations 2 and 3, substituting into equation 1 and rearranging results in the following expression:
\[
\frac{\alpha}{(F)} = K_a(1 - \alpha)
\]

A plot of \(\frac{\alpha}{(F)}\) vs. \((1 - \alpha)\) will give a linear plot with a slope of \(K_a\). All association constants were determined in this manner.

Since lumiflavin fluorescence is only slightly quenched upon binding to the protein, the association constant was determined by difference spectroscopy using the 0-0.1 absorbance unit slidewire in the Cary 14R spectrophotometer. A tandem cell arrangement was used to correct for any possible light scattering from the added protein. Absorbance changes at 432 nm were monitored to calculate the amount of bound lumiflavin. The difference in extinction between the bound and unbound lumiflavin was determined by adding a five-fold molar excess of protein.

**Preparation of Semiquinone Form of Flavoenzymes**

The Shethna flavoprotein can be isolated anaerobically in the semiquinone form. Preliminary experiments indicated no change in spectral properties of the semiquinone form prepared by light irradiation compared to those of the naturally-reduced material.

Flavoprotein free-radicals were ordinarily prepared by illumination of the samples in the presence of EDTA, according to the method of Massey and Palmer (1966). In all photoreduction experiments, the EDTA concentration was 0.05M. The enzyme solutions were made anaerobic in a
Thunberg cuvette by alternate evacuation and purging with oxygen-free nitrogen. The vacuum pump, nitrogen line, and cuvette were connected by vacuum tubing and a three-way stopcock. After approximately six cycles of evacuation and flushing, the enzyme solution was left under a slight positive pressure of nitrogen.

Illumination was carried out at 4°C in a cold room with a 150W tungsten lamp approximately 20 cm. from the sample. The sample was kept cold by circulating water through a small glass dewar from a reservoir in the cold room. This apparatus was used to measure all rates of photoreduction. In some instances where a more rapid photoreduction was desired, the sample was placed in a beaker of ice water and illuminated with a 625W Sylvania "Sun Gun" lamp at a distance of approximately 30 cm. A heat filter and two focusing lenses were used in this apparatus.

Since the photoreduction of the Shethna flavoprotein was quite slow, a method was devised for a more rapid production of semiquinone. Semiquinone formation was complete in about 12 hours when the flavoprotein was irradiated in the presence of 2M (NH₄)₂SO₄ and 0.05M EDTA at pH = 7.0. After reduction, the sample was desalted by passing through a Sephadex G-10 column (2 x 25 cm.) made anaerobic by equilibration and elution with nitrogen-purged buffer. No differences were noted in the properties
of the semiquinone prepared in this way or as described above.
RESULTS

Flavin-protein interactions in the Shethna flavoprotein have been examined using a variety of techniques. To provide a basis for the investigation, the chemical and physical properties of the protein moiety were determined (amino acid composition, molecular weight, spectra). Circular dichroism spectroscopy was employed to monitor protein conformation, the electronic transitions of the flavin chromophore, and the flavin environment on the protein. The effects of modifications of the flavin molecule on the protein-flavin interactions were studied by determining association constants, spectroscopic changes, semiquinone redox and spectral properties, and kinetics of binding. The possible role of tyrosyl and tryptophyl residues in interactions with flavin were investigated using spectroscopy and tyrosyl ionization behavior as a function of flavin binding and redox state. Some studies of CD spectra and redox properties of a variety of other flavoproteins were also performed.

Circular Dichroism Studies of the
Flavin Chromophore

Circular dichroism spectroscopy (CD) is a valuable tool in resolving electronic transitions which cannot be observed in ordinary absorption spectroscopy. This is
because of the involvement, in determining the rotational strength of a transition, of the component of the magnetic transition dipole which is non-orthogonal to the electric transition dipole, whereas ordinary absorption spectral intensities are determined only by the electric dipole moment. Hence, a magnetically allowed but electric-dipole forbidden electronic transition will be intense in the CD spectrum but not in the absorption spectrum. (For a general review of optical activity, the reader is referred to Eyring et al., 1963 or to Mason, 1963.)

Since the isoalloxazine ring is optically inactive, any CD bands observed in the visible region of the spectrum are due to the asymmetric environment provided by the optically active ribityl side chain and, in the case of flavoenzymes, also to the asymmetric environment of the protein moiety. Thus, CD spectra can provide structural and conformational information. Due to a high degree of conformational mobility of the side chain, the CD spectra observed with unbound FMN and riboflavin probably reflect an average of the various interactions which can occur between the ribityl side chain and the isoalloxazine ring. This permits the simultaneous occurrence of both positive and negative dichroic bands for the same transition and is probably responsible for the weak CD bands which are observed (Tollin, 1968a; Miles and Urry, 1968). This low rotational strength (particularly in the 450 nm region) and
overlapping of bands makes a definitive analysis quite difficult.

An increase in rotational strength might be expected to occur if the mobility of the flavin side chain were decreased or if the flavin molecule were rigidly held in an asymmetric environment, such as in its binding site on a protein. For example, the rotational strength of tryptophan transitions in chymotrypsin is greater than that of the CD bands of tryptophyl analogs in solution at room temperature (Strickland, Horwitz, and Billups, 1969).

**CD and Absorption Spectra of Unbound Flavins**

The low solubility of most flavin derivatives in non-polar solvents makes it difficult to use solvent shifts as an aid in spectral interpretation. However, acetylation of the side-chain hydroxyl groups eliminates this handicap. In Figures 4 and 5 are shown the CD and absorption spectra of tetra-O-acetyl riboflavin in dichloroethane and in aqueous buffer. Note that the resolution of the 450 nm band is quite apparent in the CD spectrum as well as in the absorption spectrum, when dichloroethane is the solvent. This resolution is lost and the bands shift to the red in phosphate buffer.

The CD spectra of riboflavin (Fig. 6) and of FMN (Fig. 7) are less intense but otherwise quite similar to that of tetra-O-acetyl riboflavin in aqueous solution. The most notable difference is that the apparent positive 335 nm
Fig. 4. Resolved Absorption and CD Spectra of Tetra-0-acetyl Riboflavin in 1, 2-dichloroethane.
Fig. 5. Resolved Absorption and CD Spectra of Tetra-0-acetyl Riboflavin in 0.025M Phosphate Buffer (pH = 7.0).
Fig. 6. Resolved Absorption and CD Spectra of Riboflavin in 0.1M Phosphate Buffer (pH = 7.0).
Fig. 7. Resolved Absorption and CD Spectra of FMN in 0.1M Phosphate Buffer (pH = 7.0)
dichroic band observed in FMN and in riboflavin is shifted to 360 nm in the tetra-O-acetyl derivative. This must reflect a change in the nature of the interaction between the side chain and the isoalloxazine ring upon substitution of the side-chain hydroxyl groups. Small differences are also observed between the CD spectra of riboflavin and FMN. This has also been noted by Miles and Urry (1963) and can be attributed to an effect of the terminal phosphate on the side chain-ring interactions.

To provide a better comparison of the spectral differences, the absorption and the CD spectra of the different analogs were resolved into a minimum number of Gaussian functions. By simultaneously fitting both the absorption and CD curves, varying only the sign and intensity of the individual functions, it was possible to reproduce all of the spectra in the 300-500 nm region with six functions. Such a resolution cannot separate overlapping bands which have similar polarizations (Miles and Urry, 1968) and thus the six bands represent a minimum number of transitions. Additional error is introduced into the analysis by the relative broadness of the observed spectra which limits the accuracy with which band positions can be established. The confidence in the resolution is increased by the fact that it is possible to fit the more complex CD and absorption spectra of the protein-bound flavins using a similar set of functions (see below). Since the absorption and CD spectra
can be matched with the same set of Gaussian functions, solvation effects which could shift the CD bands relative to the absorption bands (Moscowitz, Wellman, and Djerassi, 1963) do not seem to be operative. The resolutions obtained by this analysis are shown by the dashed curves in the figures.

The resolved spectra of tetra-O-acetyl riboflavin in dichloroethane and in phosphate buffer show that all of the vibronic bands are shifted 10-15 nm to the blue in the non-polar solvent. No evidence for an n→π* transition in this spectral range is obtained; the spectral shifts can all be ascribed to solvation effects on the π→π* transitions. An analysis of the band separations suggests that bands I, IV, and VI are 0-0 transitions (Table V). Such an assignment is in agreement with theoretical calculations which predict three π→π* transitions in this spectral range (Fox et al., 1967). It should be pointed out that the accuracy of the band positions represented is estimated to be approximately ±200 cm⁻¹.

Analysis of the separations of bands II, III, and V indicate a vibronic origin (Table V). Band II is assigned as a vibronic transition associated with the lowest energy 0-0 band. Band III is also assigned as a vibronic overtone of the lowest energy 0-0 transition. That this is probably not a progression of the first vibronic band (band II) is indicated by the fact that it changes sign relative to the
TABLE V
The Resolved Spectral Bands of Free and Protein-bound Flavins

<table>
<thead>
<tr>
<th>Flavin</th>
<th>Band I</th>
<th>Band II</th>
<th>Band III</th>
<th>Band IV</th>
<th>Band V</th>
<th>Band VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-O-acetyl riboflavin in dichloroethane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4650Å</td>
<td>4400Å</td>
<td>4130Å</td>
<td>3680Å</td>
<td>3550Å</td>
<td>3320Å</td>
</tr>
<tr>
<td></td>
<td>0-0</td>
<td>0+1200 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>0+1200+1500 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>0-0</td>
<td>0+1000</td>
<td>0-0</td>
</tr>
<tr>
<td>tetra-O-acetyl riboflavin in 0.025M phosphate pH 7.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4750</td>
<td>4490</td>
<td>4210</td>
<td>3820</td>
<td>3700</td>
<td>3450</td>
</tr>
<tr>
<td></td>
<td>0-0</td>
<td>0+1200</td>
<td>0+1200+1500</td>
<td>0-0</td>
<td>0+1000</td>
<td>0-0</td>
</tr>
<tr>
<td>FHII in 0.1M phosphate pH 7.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4730</td>
<td>4480</td>
<td>4180</td>
<td>3820</td>
<td>3600</td>
<td>3350</td>
</tr>
<tr>
<td></td>
<td>0-0</td>
<td>0+1200</td>
<td>0+1200+1600</td>
<td>0-0</td>
<td>0+1600</td>
<td>0-0</td>
</tr>
<tr>
<td>riboflavin in 0.1M phosphate pH 7.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4700</td>
<td>4490</td>
<td>4200</td>
<td>3810</td>
<td>3590</td>
<td>3340</td>
</tr>
<tr>
<td></td>
<td>0-0</td>
<td>0+1200</td>
<td>0+1200+1500</td>
<td>0-0</td>
<td>0+1600</td>
<td>0-0</td>
</tr>
<tr>
<td>Shethna flavo-protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4750</td>
<td>4460</td>
<td>4230</td>
<td>3780</td>
<td>3610</td>
<td>3410</td>
</tr>
<tr>
<td></td>
<td>0-0</td>
<td>0+1400</td>
<td>0+1400+1200</td>
<td>0-0</td>
<td>0+1200</td>
<td>0-0</td>
</tr>
<tr>
<td>deoxyFHII-Shethna apoprotein complex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4690</td>
<td>4420</td>
<td>4210</td>
<td>3720</td>
<td>3610</td>
<td>3400</td>
</tr>
<tr>
<td></td>
<td>0-0</td>
<td>0+1300</td>
<td>0+1300+1100</td>
<td>0-0</td>
<td>0+800</td>
<td>0-0</td>
</tr>
<tr>
<td>C. pasteurianum flavodoxin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4720</td>
<td>4430</td>
<td>4250</td>
<td>3800</td>
<td>3610</td>
<td>3400</td>
</tr>
<tr>
<td></td>
<td>0-0</td>
<td>0+1400</td>
<td>0+1400+1000</td>
<td>0-0</td>
<td>0+1400</td>
<td>0-0</td>
</tr>
</tbody>
</table>

*0-0 refers to the electronic transition from the 0 vibrational level in the ground state to the vibrational level in the excited state. Similarly, 0+1200 cm<sup>-1</sup> indicates a transition from the 0 vibrational level in the ground state to a 1200 cm<sup>-1</sup> vibrational level in the excited state. 0+1200+1500 cm<sup>-1</sup> denotes a transition from the 0 vibrational level in the ground state to a 1200+1500 cm<sup>-1</sup> vibrational level in the excited state (Suzuki, 1967).*
lowest energy 0-0 band in the CD spectrum of the Shethna flavoprotein. (See Fig. 8, p. 70.) This would suggest that it belongs to a vibrational mode of different symmetry than band II. The assignment of band III as vibronic and not as a separate electronic transition is based on the following considerations: (1) theoretical calculations (Fox et al., 1967; Song, 1969) do not predict another \( \pi \rightarrow \pi^* \) transition in this region of the spectrum; (2) the spectra of tetra-O-acetyl riboflavin in polar and in non-polar solvents (Figs. 4 and 5) do not provide evidence for an \( n \rightarrow \pi^* \) transition; and (3) recent theoretical work (Caldwell, 1968) predicts the possibility that a vibronic band can have a different sign from the 0-0 band in the CD spectrum. This phenomenon has also been observed in low-temperature CD studies of phenylalanine and its derivatives (Horwitz, Strickland, and Billups, 1969).

The energy spacing (Table V) is consistent with an assignment of band V as a vibronic transition associated with the second 0-0 band (band IV). On the basis of the same arguments presented for band III (see above), it seems unlikely that this is a separate electronic transition. It is noteworthy that band V has the same sign as the 0-0 band in the CD spectra of the flavoproteins (see below) and of FMN (Fig. 7) but is of opposite sign in the CD spectra of tetra-O-acetyl riboflavin (Figs. 4 and 5). This band is so weak in the CD spectrum of riboflavin (Fig. 6) that its
sign cannot be unambiguously determined. The large range in energy spacing relative to the 0-0 band (Table V) which is observed for band V could be due to the inherent error in resolving the broad featureless 370 nm spectral region. Another possibility is that the vibration excited in this transition involves the side chain-isoalloxazine ring interaction, since a significantly smaller spacing is observed in tetra-O-acetylriboflavin and the deoxyFMN-protein derivatives (Table V).

The band occurring in the spectra at 330-340 nm (band VI) is assigned as the third 0-0 transition. This is based on the molecular orbital calculations (Fox et al., 1967) and on CD and MCD studies (Tollin, 1968a). The energy spacing (2000 cm$^{-1}$) seems too large for it to be a vibronic overtone of the 375 nm transition. On the basis of the solvent studies presented, this band must be considered as resulting from a $\pi \rightarrow \pi^*$ transition, rather than from an $n \rightarrow \pi^*$ transition as suggested by Miles and Urry (1968). The fact that a transition in this spectral region is not observed in fluorescence polarization measurements could indicate that the angle between the transition moment and the emission oscillator is similar to that for the 375 nm transition.

**CD and Absorption Spectra of Protein-bound Flavin**

The changes in the absorption spectrum of flavin when it is bound to protein are small compared to the large
changes which are observed in the CD spectra (Figs. 8, 9, and 10). The flavoproteins which we are considering here all have one FMN per protein molecule, are metal-free, and have only a single polypeptide chain. This assures that there are no interfering effects from metal chromophores, from flavin-flavin interactions, or from protein subunit interactions.

As mentioned previously, resolution of the CD and absorption spectra of these flavoenzymes was achieved using six Gaussian functions, as was the case with the unbound flavins. The negative band observed at 310 nm (Figs. 8, 9, and 10) was not included in the analysis due to overlap by the strong positive dichroic bands of the aromatic amino acid occurring below 300 nm. This dichroic band could originate either from the flavin chromophore (see below) or from a tryptophyl residue. A negative dichroic band at 303 nm has been observed for chymotrypsinogen (Strickland et al., 1969) and ascribed to an indole transition. The energy spacings between the resolved bands follow the same pattern as was observed with the unbound flavins (Table V). The bands are therefore assigned as discussed above.

A comparison of the rotational strengths of the CD bands of free FMN with those for FMN bound to the Shethna flavoprotein indicates that the 0-0 transitions are increased from five- to twenty-fold (Table VI). In contrast, the rotational strengths of the vibronic bands are increased to a lesser extent. The FMN bands in C. pasteurianum
Fig. 8. Resolved CD and Absorption Spectra of the Shethna Flavoprotein.

The spectra were measured on protein solutions in 0.025M phosphate—0.05M EDTA, pH = 7.0, buffer.
Fig. 9. Resolved Absorption and CD Spectra of *C. pasteurianum* Flavodoxin.

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA, pH = 7.0, buffer.
Fig. 10. Resolved CD and Absorption Spectra of the DeoxyFMN-Shethna Apoprotein Complex.

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA, pH = 7.0, buffer.
<table>
<thead>
<tr>
<th>Flavin</th>
<th>Band</th>
<th>( f_1 )</th>
<th>( R_4 \times 10^4 )</th>
<th>( f_1 )</th>
<th>( R_4 \times 10^4 )</th>
<th>( f_1 )</th>
<th>( R_4 \times 10^4 )</th>
<th>( f_1 )</th>
<th>( R_4 \times 10^4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetra-O-acetyl riboflavin in dichloroethane</td>
<td>I</td>
<td>0.046</td>
<td>-1.8</td>
<td>0.039</td>
<td>-1.4</td>
<td>0.099</td>
<td>-3.7</td>
<td>0.025</td>
<td>-4.5</td>
</tr>
<tr>
<td>tetra-O-acetyl riboflavin in 0.025M phosphate pH = 7.0</td>
<td>II</td>
<td>0.055</td>
<td>-3.1</td>
<td>0.044</td>
<td>-2.2</td>
<td>0.089</td>
<td>-5.1</td>
<td>0.015</td>
<td>-0.8</td>
</tr>
<tr>
<td>FCII in 0.1M phosphate pH = 7.0</td>
<td>III</td>
<td>0.049</td>
<td>-0.8</td>
<td>0.060</td>
<td>-0.7</td>
<td>0.064</td>
<td>-1.1</td>
<td>0.050</td>
<td>-0.5</td>
</tr>
<tr>
<td>riboflavin 0.1M phosphate pH = 7.0</td>
<td>IV</td>
<td>0.068</td>
<td>-1.4</td>
<td>0.053</td>
<td>-0.7</td>
<td>0.080</td>
<td>-1.2</td>
<td>0.073</td>
<td>0</td>
</tr>
<tr>
<td>Shethna flavoprotein</td>
<td>V</td>
<td>0.067</td>
<td>+10.5</td>
<td>0.029</td>
<td>+2.7</td>
<td>0.080</td>
<td>-5.3</td>
<td>0.061</td>
<td>-25.4</td>
</tr>
<tr>
<td>deoxyFCII-Shethna apoprotein complex</td>
<td>VI</td>
<td>0.083</td>
<td>+5.0</td>
<td>0.017</td>
<td>+1.3</td>
<td>0.091</td>
<td>-14.3</td>
<td>0.048</td>
<td>-22.0</td>
</tr>
</tbody>
</table>

Sh. pasteurianum flavodoxin

*The oscillator strength (\( f_1 \)) is calculated from the expression: \( f_1 = 4.32 \times 10^{-7} \epsilon_1 \Delta \lambda_1 \), where \( \epsilon_1 \) is the molar extinction at the curve maximum and \( \Delta \lambda_1 \) is the bandwidth at half-maximal intensity (Suzuki, 1967).

*The rotational strength (\( R_4 \)) is calculated from the expression: \( R_4 = 1.23 \times 10^{-42} \Theta_1 \Delta \lambda_1 \), where \( \Theta_1 \) is the molar ellipticity at the curve maximum, \( \Delta \lambda_1 \) is the bandwidth in nm at half-maximum intensity, and \( \lambda_1 \) is the wavelength in nm at maximum intensity (Miles and Urry, 1968).
flavodoxin show a somewhat different pattern of change in the rotational strengths. In addition, the sign of band III is opposite to that of the Shethna flavoprotein. The reasons for these differences are unclear. However, they must reflect differences in the environments of the FMN molecule in the two flavoproteins, even though the over-all shape of their CD spectra are quite similar (Figs. 8 and 9).

The CD spectrum of the deoxyFMN-Shethna apoprotein complex\(^1\) (Fig. 10) is a result of only protein-induced asymmetry, inasmuch as the flavin side chain has no asymmetric carbons. It is evident that the over-all shape of the spectrum is quite similar to that for the FMN protein. However, some differences are observed. One such difference is a red shift of the absorption bands of the FMN-protein complex relative to those of the deoxyFMN-protein complex (Table V). This indicates that the hydroxyl groups provide a polar environment for the isoalloxazine ring when it is bound to the protein. This spectral shift can be seen more clearly in the optical difference spectra of the bound minus unbound flavins (Fig. 11). Note also that a hypochromic effect and a "tailing" of the long wavelength band to the red are observed for both derivatives. Also significant is the weaker positive dichroism at 470 nm for the bound

---

\(^1\)The binding of flavin analogs to the Shethna apoprotein is discussed below. The CD spectrum of the FMN-Shethna apoprotein complex is identical to that of the native flavoprotein.
Fig. 11. Difference Spectra of Shethna Apoprotein-bound Flavin Minus Unbound Flavin.

- FFM-protein complex minus FFM in 0.025M phosphate, pH = 7.0
- DeoxyFFM-protein complex minus deoxyFFM in 0.025M phosphate, pH = 7.0
deoxyFMN as compared to the bound FMN. These effects are also seen with the protein-bound riboflavin and deoxyriboflavin analogs (see below).

The optical difference spectra provide insights into the environment of the flavin chromophore on the protein. As is shown in Figures 4 and 5, the absorption spectrum of flavin in a non-polar solvent is partially resolved in the long wavelength region. Little or no hypochromism is observed and no appreciable "tailing" of the 450 nm band occurs (Kotaki et al., 1967; Palmer and Massey, 1968). These latter effects, however, do occur when flavins are complexed with other aromatic molecules, e.g., with phenols (Fleischman and Tollin, 1965) or with adenine in FAD. In the flavoproteins considered here, increased resolution, hypochromism and long wavelength tailing are observed, although no blue shifts are apparent. Thus, it may be that the binding of FMN to the Shethna apoprotein, as well as that of the flavodoxins, results in placing the isoalloxazine ring in a less polar environment than in water and close to one or more aromatic amino acid residues of the protein. It is conceivable that the combination of effects accounts for the lack of a blue shift of the vibronic bands.

The CD data are also suggestive that the rotational strength of the 0-0 band in the 470 nm region is particularly sensitive to side-chain hydroxyl group interaction with the isoalloxazine ring, and that this interaction is
greatly increased in magnitude when FMN is bound to the proteins considered here. (The optical difference spectra are also consistent with this.) Such a correlation could explain the occurrence of strong optical activity in the 450 nm region of certain FAD-containing flavoenzymes, for example, p-hydroxybenzoate hydroxylase (Hesp, Calvin, and Hosokawa, 1969). Free FAD has little or no optical activity in this spectral region (Miles and Urry, 1968; Hesp et al., 1969). This may be because the ribityl side chain cannot interact strongly with the isoalloxazine ring as a result of steric limitations caused by the complexation with the adenine ring. Specific protein-FAD interactions could break up this complex and could position the ribityl side chain and the isoalloxazine ring in such a manner as to produce the observed CD spectrum. This is evident in the CD spectrum of the FAD-Shethna apoprotein complex. (See Fig. 39, p. 131.) In the case of lipoyl dehydrogenase, another FAD flavoprotein, the CD data show no optical activity in the long wavelength region (Brady and Beychok, 1969). This would suggest that the side chain-ring interactions are weak, even though the flavin-adenine complex is no longer intact, as evidenced by the increase in fluorescence of the bound FAD (Palmer and Massey, 1968).

More experiments are needed to verify this hypothesis. However, the interpretation is reinforced by the observation that FAD analogs containing no ribityl hydroxyl
groups have essentially identical absorption and fluorescence properties as does FAD (Chassey and McCormick, 1965a). A comparison of CD spectral properties of the deoxy analog with FAD would be of interest. In addition, the suggestion from CD data that there is little or no isoalloxazine ring-ribityl side chain interaction in free FAD could perhaps be more unequivocally indicated by NMR studies, since the possibility of a cancelling effect by overlapping positive and negative CD bands could be operative.

A flavin transition occurring at about 300 nm has been detected by fluorescence polarization measurements (Kurtin and Song, 1963) and by CD measurements (Miles and Urry, 1968). Because of overlapping bands, it is difficult to decide whether or not this transition appears in the CD spectra of the flavoproteins. Miles and Urry (1968) have suggested that this is an $n\rightarrow\pi^*$ transition because of its low oscillator strength. If this assignment is correct, one should observe a large red shift in going from polar to non-polar solvents. In Figure 12 is shown the CD spectra of concentrated solutions of tetra-O-acetyl riboflavin, FMN and FAD in water and in dichloroethane, along with the results of a curve resolution. The solvent polarity is seen to have only a small effect on the 300 nm transition (a small blue shift in going from water to dichloroethane). The loss in resolution which occurs in dichloroethane is almost entirely due to the large blue shift of the 383 nm band (see Table V).
Fig. 12. CD Spectra of Flavin Solutions in the Near Ultraviolet Region.

ooooo FAD (4.4 x 10^{-4}M) in H2O

----- FMN (9.4 x 10^{-4}M) in H2O

----- Tetra-O-acetyl riboflavin (11.3 x 10^{-4}M) in 1, 2-dichloroethane

----- Tetra-O-acetyl riboflavin (4.15 x 10^{-5}M) in H2O
(The ellipticity values are multiplied by three)

Ten mm pathlength cells were used for all measurements except for tetra-O-acetyl riboflavin in water in which a 50 mm pathlength cell was used. The arrows point to the measured spectra. The other curves are obtained from curve resolution into Gaussian functions for the appropriate spectrum. The FAD spectrum was not resolved.
Fig. 12. CD Spectra of Flavin Solutions in the Near Ultraviolet Region.
The transition is seen to be quite side-chain dependent, however, both in sign and in position. It is possible that effects from dimerization could be operative at the concentrations employed, although no changes were seen upon a five-fold dilution. Thus, there is presently no experimental evidence to assign this band as being due to an \( n \rightarrow \pi^* \) transition.

**A Comparison of the Circular Dichroism Spectra of a Number of Flavoenzymes**

**CD and Absorption Spectra of the Oxidized Forms**

The similar properties of the dehydrogenase flavoproteins (Massey et al., 1969a) may be a reflection of similarities in the environments of the bound flavins. Indeed, as shown above, the CD spectra of the Shethna flavoprotein and of *C. pasteurianum* flavodoxin are quite close in over-all shape, although analysis of the spectra does indicate small differences (Table VI).

The CD spectrum of *P. elsdenii* flavodoxin is nearly identical with that of *C. pasteurianum* flavodoxin (Fig. 13), while that of *R. rubrum* flavodoxin (Fig. 14) differs from the *P. elsdenii* and *C. pasteurianum* flavodoxins in some respects, although again the over-all shapes are similar. It is interesting that the flavin dichroic bands of *R. rubrum* flavodoxin are more similar to those of the Shethna flavoprotein (Fig. 8) than to the other flavodoxins. This is also reflected in redox properties (see below).
Fig. 13. Resolved CD and Absorption Spectra of *P. elsdenii* Flavodoxin in the Visible Region.

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA, pH = 7.0, buffer.
Fig. 14. Resolved CD and Absorption Spectra of *R. rubrum* Flavodoxin in the Visible Region.

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA, pH = 7.0, buffer.
Fig. 14. Resolved CD and Absorption Spectra of R. rubrum Flavodoxin in the Visible Region.
Since all of the preceding dehydrogenase flavoenzymes contain FMN, it was of interest to measure the CD spectrum of an FAD-containing dehydrogenase. In Figure 15 is shown the CD spectrum of spinach ferredoxin-TPNH reductase. It can be seen that this spectrum is quite similar to those of other dehydrogenases except that all of the bands are reversed in sign. This indicates that the flavin environment resembles that of other dehydrogenases but that the perturbations induce opposite polarizations in the flavin transitions. The spectrum also shows that the adenine-isoalloxazine complex is broken upon binding of the FAD. It is of interest to note that this flavoenzyme transfers electrons to, and forms complexes with, ferrodoxins and flavodoxins (Foust, Mayhew, and Massey, 1969).

The circular dichroism spectra of the oxidase flavoenzymes are quite different in shape from those of the dehydrogenases (Figs. 16 and 17). The most prominent dichroic band in glucose oxidase and in L-amino acid oxidase is at 380 nm. Positive dichroic bands are observed at longer wavelengths in the case of L-amino acid oxidase, but are very weak in glucose oxidase. The CD spectrum of D-amino acid oxidase (Kotaki, Sugiura, and Yagi, 1963) is quite similar to that of glucose oxidase both in band positions and in intensity.

Curve resolution of the oxidase and dehydrogenase CD and absorption spectra indicates six vibronic bands, as was
Fig. 15. Resolved Absorption of CD Spectra of Spinach Ferredoxin-TPNH Reductase in the Visible Region.

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA, pH = 7.0, buffer.
Fig. 16. Resolved CD and Absorption Spectra of Glucose Oxidase in the Visible Region.

The spectra were measured on protein solutions in 0.1M acetate--0.05M EDTA, pH = 6.0, buffer.
Fig. 17. Resolved CD and Absorption Spectra of Snake Venom L-amino Acid Oxidase (C. adamanteus) in the Visible Region.

The spectra were measured on protein solutions in 0.025M phosphate—0.05M EDTA, pH = 7.0, buffer. The enzyme from C. atrox gave essentially the same results.
found in the free flavins. Inasmuch as the spacings are quite similar (Table VII), the bands can be assigned as discussed in the section on flavin spectroscopy.

An analysis of the rotational strengths and signs of the resolved bands of the dehydrogenases indicates two general patterns (Table VIII). The second vibronic band (band III) is opposite in sign to the 0-0 band (band I) in the CD spectra of the Shethna flavoprotein, the deoxyFAD-Shethna apoprotein complex, R. rubrum flavodoxin, and ferredoxin-TPNH reductase. In contrast, band III has the same sign as the 0-0 band (band I) in the CD spectra of C. pasteurianum flavodoxin and P. elsdenii flavodoxin. The CD spectrum of the 3-methyl-FMN-Shethna apoprotein complex (Fig. 13) indicates a pattern quite similar to that of the C. pasteurianum and P. elsdenii flavodoxins, in that the sign of band III is the same as that for band I. As will be seen later, some of the redox properties of this derivative also resemble those of the flavodoxins. It is of interest to note that the semiquinone forms of those flavoproteins in which band III is of opposite sign from band I in the CD spectra (with the exception of FTR) are resistant to further reduction by dithionite at neutral pH, while those flavoenzymes in which the sign of band III is the same as that of band I are reduced to the flavin hydroquinone form. The structural significance of this apparent relationship is unclear.
# TABLE VII
The Resolved spectral Bands of the Dehydrogenases and Oxidases

| Flavoprotein                                | Band |
|---------------------------------------------|------|------|------|------|------|------|------|------|
| P. elsdenii flavodoxin                      | I    | II   | III  | IV   | V    | VI   |
|                                             | 4710Å | 4420Å | 4230Å | 3780Å | 3590Å | 3320Å |
|                                             | 0-0  | 0+1400cm⁻¹ | 0+1400+1000cm⁻¹ | 0-0 | 0+1400cm⁻¹ | 0-0 |
| R. rubrum flavodoxin                        |      |      |      |      |      |      |
|                                             | 4870 | 4570 | 4300 | 3920 | 3780 | 3500 |
|                                             | 0-0  | 0+1300 | 0+1300+1400 | 0-0 | 0+900 | 0-0 |
| ferredoxin-TPNH reductase                   |      |      |      |      |      |      |
|                                             | 4900 | 4610 | 4420 | 3850 | 3610 | 3350 |
|                                             | 0-0  | 0+1300 | 0+1300+900 | 0-0 | 0+1700 | 0-0 |
| 3-methyl-FMN-Shethna apoprotein complex     |      |      |      |      |      |      |
|                                             | 4800 | 4520 | 4320 | 3620 | 3680 | 3480 |
|                                             | 0-0  | 0+1300 | 0+1300+1000 | 0-0 | 0+1000 | 0-0 |
| glucose oxidase                             |      |      |      |      |      |      |
|                                             | 4310 | 4510 | 4210 | 3870 | 3610 | 3400 |
|                                             | 0-0  | 0+1400 | 0+1400+1400 | 0-0 | 0+800 | 0-0 |
| L-amino acid oxidase                        |      |      |      |      |      |      |
|                                             | 4970 | 4650 | 4380 | 4050 | 3910 | 362 |
|                                             | 0-0  | 0+1400 | 0+1400+1300 | 0-0 | 0+900 | 0-0 |

Note: The band designations are as described in Table V.
### TABLE VIII

Intensities of the Resolved Spectral Bands of the Dehydrogenases and Oxidases

<table>
<thead>
<tr>
<th>Band</th>
<th>Flavoprotein</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$f_1$</td>
<td>$R_{1\times 10^{40}}$</td>
<td>$f_1$</td>
<td>$R_{1\times 10^{40}}$</td>
<td>$f_1$</td>
<td>$R_{1\times 10^{40}}$</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>0.053</td>
<td>+10.4</td>
<td>0.023</td>
<td>+4.7</td>
<td>0.097</td>
<td>+7.2</td>
</tr>
<tr>
<td>II</td>
<td>P. elsdonii flavodoxin</td>
<td>0.070</td>
<td>+25.9</td>
<td>0.026</td>
<td>+7.7</td>
<td>0.093</td>
<td>-3.6</td>
</tr>
<tr>
<td>III</td>
<td>R. rubrum flavodoxin</td>
<td>0.025</td>
<td>-6.1</td>
<td>0.023</td>
<td>-6.1</td>
<td>0.127</td>
<td>+6.1</td>
</tr>
<tr>
<td>IV</td>
<td>ferredoxin-TPNH reductase</td>
<td>0.031</td>
<td>+6.1</td>
<td>0.013</td>
<td>+3.3</td>
<td>0.092</td>
<td>+4.3</td>
</tr>
<tr>
<td>V</td>
<td>3-methyl-FMN-Shethna apoprotein complex</td>
<td>0.064</td>
<td>0</td>
<td>0.079</td>
<td>+1</td>
<td>0.090</td>
<td>+4.6</td>
</tr>
<tr>
<td>VI</td>
<td>glucose oxidase</td>
<td>0.044</td>
<td>+11.2</td>
<td>0.065</td>
<td>+16.8</td>
<td>0.074</td>
<td>+17.2</td>
</tr>
</tbody>
</table>

Note: The definition of $f_1$ and $R_1$ are as described in Table VI.
Fig. 18. Resolved CD and Absorption Spectra of the 3-methylFKN-Shethna Apoprotein Complex in the Visible Region.

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA buffer, pH = 7.0.
A second pattern which is observed is that the third 0-0 transition (band VI) is of comparatively low rotational strength in the *C. pasteurianum* and *P. elsdenii* flavodoxins, but has a large rotational strength in *R. rubrum* flavodoxin, ferredoxin-TPNH reductase, the 3-methyl-FMN- and the deoxyFMN-Shethna apoprotein complexes and in the Shethna flavoprotein. The 3-methyl-FMN-Shethna apoprotein complex therefore does not completely follow the CD properties of the *C. pasteurianum* and *P. elsdenii* flavodoxins.

The CD spectrum of the isoFMN-Shethna apoprotein complex (Fig. 19) is quite similar in shape and intensity to that of the native Shethna flavoprotein. The slight red shift of the 476 nm positive CD band to 488 nm is also apparent in the absorption spectrum. Since the flavin transitions are modified by the methyl shift from the 8 to the 6 position, a direct comparison of the individual bands cannot be made. However, the loss of the methyl group at the 8 position and the methyl addition to the 6 position does not appreciably alter the environment of the bound flavin.

The most notable feature of the oxidase CD spectra is the lack of any measurable rotational strength band VI. The low rotational strength in the 450 nm region in the case of glucose oxidase (Fig. 16) could indicate the absence of side chain-isoalloxazine ring interaction as discussed in the section on flavin spectroscopy. On the other hand, the CD spectrum of L-amino acid oxidase (Fig. 17) indicates that
Fig. 19. CD and Absorption Spectra of the IsoFMN-Shethna Apoprotein Complex in the Visible Region.

----- Absorption spectrum

----- CD spectrum

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA buffer, pH = 7.0.
Fig. 19. CD and Absorption Spectra of the IsoFMN-Shethna Apoprotein Complex in the Visible Region.
side chain-flavin ring interaction does occur in this protein. Both oxidase flavoenzymes contain FAD as the flavin group and have two FAD molecules per molecule of protein (Swoboda and Massey, 1965; Wellner and Meister, 1960).

**CD and Absorption Spectra of the Semiquinone Forms of Flavoenzymes**

To further compare the flavin environments in the dehydrogenases and oxidases, the CD and absorption spectra of the flavin semiquinone forms were measured. Figure 20 shows the CD and absorption spectra of the semiquinone form of the Shethna flavoprotein. The CD spectrum follows the absorption spectrum except for the large negative dichroic band at 500 nm which has a low extinction in the absorption spectrum and a lack of optical activity in the 400-450 nm region. The CD and absorption spectra of the semiquinone forms of the R. rubrum flavodoxin (Fig. 21) and the 3-methyl-FMN Shethna apoprotein complex (Fig. 22) are quite similar to those of the Shethna flavoprotein. The semiquinone form of the deoxyFMN Shethna apoprotein complex is also quite similar except for the rotational strength of the two bands in the 600 nm region (Fig. 23). Thus, the longest wavelength region of the spectrum again reflects side chain-ring interaction. This decrease of rotational strength is apparent in the CD spectrum of the isoFMN-Shethna apoprotein complex (Fig. 24). The absorption spectrum of this complex
Fig. 20. Absorption and CD Spectra of the Semiquinone Form of the Shethna Flavoprotein in the Visible Region.

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA buffer, pH = 7.0.

CD spectrum

Absorption spectrum

Note that the absorption spectrum is inverted to facilitate comparison.
Fig. 20. Absorption and CD Spectra of the Semiquinone Form of the Shethna Flavoprotein in the Visible Region.
Fig. 21. Absorption and CD Spectra of the Semiquinone Form of *R. rubrum* Flavodoxin in the Visible Region.

The spectra were measured and are as indicated in Figure 20.
Fig. 22. CD and Absorption Spectra of the Semiquinone Form of the 3-methylFMN-Shethna Apoprotein Complex in the Visible Region.

The spectra were measured and are as indicated in Figure 20.
Fig. 23. CD and Absorption Spectra of the Semiquinone Form of the DeoxyFMN-Shethna Apoprotein Complex in the Visible Region.

The spectra were measured and are as indicated in Figure 20.
Fig. 24. CD and Absorption Spectra of the Semiquinone Form of the IsoFMN-Shethna Apoprotein Complex in the Visible Region.

The spectra were measured and are as indicated in Figure 20.
is also appreciably different from those of the previous flavoenzyme semiquinones.

The absorption characteristics of the *C. pasteurianum* (Fig. 25) and the *P. elsdenii* (Fig. 26) semiquinones are more similar to those of the isoFMN semiquinone, even though both of these flavoenzymes have FMN as their flavin chromophore. The CD spectra of these two semiquinones are almost identical, and also resemble the spectra of the semiquinone forms of the other dehydrogenases. The small differences which are observed are a further verification of the minor differences in flavin environment found for the oxidized flavoenzyme dehydrogenases.

No curve analysis was done on the semiquinone spectra since it is technically impossible to determine the CD spectrum of unbound flavin radical due to the high rate of disproportionation. Also, to date, there are no molecular orbital calculations on flavin semiquinones. The spectral properties of the ferredoxin-TPNH reductase semiquinone form are not presented due to the difficulty in preparing substantial amounts of the half-reduced form.

Since both the neutral (blue) and anionic (red) semiquinone forms of glucose oxidase can be prepared (Massey and Palmer, 1966), this flavoenzyme provides a "bridge" for comparing the semiquinone environments of the dehydrogenases and oxidases. The absorption spectrum of the neutral semiquinone form of glucose oxidase is similar
Fig. 25. CD and Absorption Spectra of the Semiquinone Form of *C. pasteurianum* Flavodoxin in the Visible Region.

The spectra were measured and are as indicated in Figure 20.
Fig. 26. CD and Absorption Spectra of the Semiquinone Form of P. elsdenii Flavodoxin in the Visible Region.

The spectra were measured and are as indicated in Figure 20.
to that of the dehydrogenase flavoenzymes (Fig. 27). The CD spectrum, however, is quite different, as evidenced principally by the lack of optical activity at energies lower than 400 nm. The positive band at 375 nm is about one-third the intensity of the negative 355 nm band in the Shethna flavoprotein. The negative band at 310 nm appears to be an artifact resulting from irradiation, since it does not disappear upon reoxidation. (In this respect, the CD spectra of all of the other flavoproteins were identical upon reoxidation to those of the non-irradiated samples.) The nature of this irreversible effect is not known. The absorption spectrum of the reoxidized material is identical to the non-irradiated flavoenzyme and no enzymatic activity is lost (Massey and Palmer, 1966).

The absorption spectrum of the anionic glucose oxidase semiquinone (Fig. 28) is similar to that of the semiquinone of L-amino acid oxidase (Fig. 29), although the very sharp band at 400 nm in glucose oxidase does not appear in the L-amino acid oxidase spectrum. This band is the major CD absorption in glucose oxidase (Fig. 28), while the 370 nm maximum observed in the amino acid oxidase spectrum can be identified as a shoulder on the 400 nm CD band. The CD spectrum of the L-amino acid oxidase anionic semiquinone has a major band at 400 nm which seems to be a component of the asymmetric 395 nm absorption maximum. The broad "tail" in the CD spectra of both oxidase
Fig. 27. CD and Absorption Spectra of the Neutral Semiquinone Form of Glucose Oxidase in the Visible Region.

- Absorption spectrum
- CD spectrum
- CD spectrum of reoxidized material

All spectra were measured on protein solutions in 0.1M acetate—0.05M EDTA buffer, pH = 6.0.
Fig. 27. CD and Absorption Spectra of the Neutral Semiquinone Form of Glucose Oxidase in the Visible Region.
Fig. 28. CD and Absorption Spectra of the Anionic Semiquinone Form of Glucose Oxidase in the Visible Region.

--- Absorption spectrum
----- CD spectrum
------- CD spectrum of non-irradiated material
......... CD spectrum of reoxidized material

All spectra were measured on protein solutions in 0.1M glycine--0.05M EDTA buffer, pH = 9.5.
Fig. 28. CD and Absorption Spectra of the Anionic Semiquinone Form of Glucose Oxidase in the Visible Region.
Fig. 29. CD and Absorption Spectra of the Anionic Semiquinone Form of (*C. adamantaeus*) L-amino Acid Oxidase in the Visible Region.

--- Absorption spectrum

--- CD spectrum

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA buffer, pH = 7.0. The *C. atrox* enzyme gave essentially the same results.
semiquinones in the 450-500 nm region reflects the 490 nm band apparent in both absorption spectra. As was observed at pH = 6.0, an irreversible change occurs in the photoreduction of glucose oxidase at pH = 9.5. This can be seen by comparing the CD spectrum of the reoxidized material with the nonphotoreduced material (Fig. 28).

The CD spectra of other flavoenzymes in their oxidized forms: egg white flavoprotein (Nishikimi and Yagi, 1969), p-hydroxy benzoate hydroxylase (Hesp et al., 1969), and lipoyl dehydrogenase (Brady and Beychok, 1969), all are fairly similar in shape to the dehydrogenases. The egg white flavoprotein has no known redox function but forms a blue semiquinone on dithionite titration (Nishikimi and Yagi, 1969). Lipoyl dehydrogenase forms a non-paramagnetic long wavelength absorbing species (Palmer and Massey, 1968). A redox active disulfide bond is also implicated in its catalytic mechanism so that a direct comparison with the dehydrogenases studied here would not be proper. No semiquinone form was detected in the dithionite titration of p-hydroxybenzoate hydroxylase (Hesp et al., 1969).

The above results demonstrate quite clearly that, as one might expect a priori, flavin environment is a key determinant in redox behavior of flavoenzymes and that CD spectroscopy provides the enzymologist with a powerful tool for investigating certain aspects of flavin conformation in flavoproteins.
Chemical and Physical Properties of the Shethna Flavoprotein

Molecular Weight

The sedimentation coefficient of the Shethna flavoprotein was determined at five different protein concentrations ranging from 4-8 mg/ml. The protein samples gave only a single, symmetric refractive index gradient. As shown in Figure 30, a plot of ln x vs. time is linear, where x is the distance in centimeters from the center of the boundary to the axis of rotation. Table IX indicates very little concentration dependence of the value of the sedimentation coefficient ($S_{20,w}$). The average of the five determinations gave a value for $S_{20,w}$ of $2.33 \times 10^{-13}$ sec.

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>$S_{20,w}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>2.39</td>
</tr>
<tr>
<td>7.0</td>
<td>2.33</td>
</tr>
<tr>
<td>6.5</td>
<td>2.33</td>
</tr>
<tr>
<td>5.3</td>
<td>2.36</td>
</tr>
<tr>
<td>4.0</td>
<td>2.30</td>
</tr>
</tbody>
</table>

The diffusion coefficient was determined at three different protein concentrations. As is shown in Figure 31, a
Fig. 30. Sedimentation of the Shethna Flavoprotein as a Function of Time.

The protein concentration is 5.3 mg/ml in 0.025M phosphate buffer, pH = 7.0.
Fig. 31. Plot of $A^2/H^2$ versus Time in the Determination of the Diffusion Coefficient of the Shethna Flavoprotein.

The protein concentration is 3.47 mg/ml in 0.025M phosphate buffer, pH = 7.0.
plot of $A^2/H^2$ vs. time is linear, where $A$ is the area under the schlieren peak and $H$ is maximum height of the peak at time $t$. The diffusion coefficient did vary with concentration as is indicated by Figure 32. The diffusion coefficient ($D_{20,w}$), corrected to infinite dilution, is $9.03 \times 10^{-7} \text{cm}^2\text{-sec}^{-1}$.

The molecular weight of the Shethna flavoprotein was calculated using the determined values of the diffusion coefficient and the sedimentation coefficient and a value for the partial specific volume (0.723) which was calculated from the amino acid composition (Cohn and Edsall, 1943). Using the Svedberg equation (given in the Experimental section), the molecular weight was calculated to be 22,600 g-mole$^{-1}$.

The molecular weight was also determined by sedimentation equilibrium using the short column technique of Yphantis (1964). As shown in Figure 33, a plot of $\ln y/r$ vs. $r^2$ was linear, where $r$ is the distance in centimeters from the refractive index increment to the axis of rotation and $y$ is height of the refractive index gradient at distance $r$. The molecular weight values determined at protein concentrations of 2.3, 3.5, and 7 mg/ml were 26,500, 24,300, and 23,600 g-mole$^{-1}$, respectively. An average of the three determinations gave a molecular weight for the Shethna flavoprotein of 24,800 g-mole$^{-1}$. 
Fig. 32. The Variation of the Diffusion Coefficient of the Shethna Flavoprotein with Concentration.

The variation of the diffusion coefficient of the Shethna flavoprotein with concentration.
Fig. 33. Plot of \( \ln \frac{y}{r} \) versus \( r^2 \) in the Determination of the Molecular Weight of the Shethna Flavoprotein by Sedimentation Equilibrium.

The protein concentration is 3.5 mg/ml in 0.025M phosphate buffer, pH = 7.0.
Fig. 33. Plot of ln \( y/r \) versus \( r^2 \) in the Determination of the Molecular Weight of the Shethna Flavoprotein by Sedimentation Equilibrium.
The amino acid composition (see below) gives a value of 21,300 g-mole\(^{-1}\). This is calculated from the combined weight of the substituent amino acids minus the water contribution. The weight of amide ammonia was not included, inasmuch as the amide content has not been determined. The average of the three methods gives a molecular weight of 23,000 g-mole\(^{-1}\) for the Shethna flavoprotein.

**Amino Acid Composition**

The amino acid analysis of the Shethna flavoprotein and the apoprotein were identical (as indicated by Table X). Assuming one mole of cysteine per mole of protein, the number of residues of each amino acid per mole was calculated. Since the calculated molecular weight is similar to the molecular weight determined by hydrodynamic measurements (see above) the assumption of one mole of cysteine per mole of protein seems justified. Independent methods of tryptophan determination (Table X) also gave the same value as the one based on the total amino acid analysis.

The amino acid composition data indicate the absence of histidine and cystine, four tryptophans, five tyrosines, and a high percentage of glutamic acid, aspartic acid, and glycine. The composition determined for the Shethna protein is similar to the published amino acid compositions of *P. elsdenii* flavodoxin (Hayhew and Massey, 1969) and *C. pasteurianum* flavodoxin (Knight and Hardy, 1967). These two flavodoxins also have no histidine or cystine
TABLE X

The Amino Acid Composition of the Shethna Flavoprotein

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Apoprotein Micromoles</th>
<th>Flavoprotein Micromoles</th>
<th>Residues Mole Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>.173</td>
<td>.173</td>
<td>13</td>
</tr>
<tr>
<td>Histidine</td>
<td>.001</td>
<td>.003</td>
<td>0</td>
</tr>
<tr>
<td>Arginine</td>
<td>.078</td>
<td>.075</td>
<td>6</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>.263</td>
<td>.270</td>
<td>21</td>
</tr>
<tr>
<td>Asparagine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>.107</td>
<td>.104</td>
<td>8</td>
</tr>
<tr>
<td>Serine</td>
<td>.190</td>
<td>.189</td>
<td>15</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>.293</td>
<td>.294</td>
<td>23</td>
</tr>
<tr>
<td>Glutamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>.072</td>
<td>.069</td>
<td>5</td>
</tr>
<tr>
<td>Glycine</td>
<td>.270</td>
<td>.270</td>
<td>21</td>
</tr>
<tr>
<td>Alanine</td>
<td>.193</td>
<td>.196</td>
<td>15</td>
</tr>
<tr>
<td>Valine</td>
<td>.127</td>
<td>.125</td>
<td>10</td>
</tr>
<tr>
<td>Methionine</td>
<td>.015</td>
<td>.013</td>
<td>1</td>
</tr>
<tr>
<td>Iso Leucine</td>
<td>.077</td>
<td>.074</td>
<td>6</td>
</tr>
<tr>
<td>Leucine</td>
<td>.272</td>
<td>.258</td>
<td>20</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>.070</td>
<td>.069</td>
<td>5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>.177</td>
<td>.168</td>
<td>13</td>
</tr>
<tr>
<td>1/2-cystine</td>
<td>.013</td>
<td>.013</td>
<td></td>
</tr>
<tr>
<td>Cysteic Acid</td>
<td>.014</td>
<td>.016</td>
<td>1</td>
</tr>
<tr>
<td>Carboxymethyl Cysteine</td>
<td>.010</td>
<td>.013</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>({Ba/ OH/2 hydrolysis})</td>
<td>.053</td>
<td>.058</td>
<td>1</td>
</tr>
</tbody>
</table>
### TABLE X—Continued

<table>
<thead>
<tr>
<th>Tryptophan</th>
<th>Spectrophotometric Titration</th>
<th>3.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan</td>
<td>N-bromosuccinimide Oxidation</td>
<td>3.7</td>
</tr>
</tbody>
</table>

and contain four tryptophans. *C. pasteurianum* flavodoxin contains one cysteine while *P. elsdenii* flavodoxin contains two cysteines. The similarities in the compositions of the three flavoproteins are quite striking, considering that the molecular weights of the two flavodoxins are 15,000 g-mole\(^{-1}\) as compared to 23,000 g-mole\(^{-1}\) for the Shethna flavoprotein. All three flavoproteins contain one FMN per mole of protein (Knight and Hardy, 1967; Mayhew and Massey, 1969; Hinkson and Bulen, 1967). The Shethna flavoprotein, in spite of its similar chemical composition, does not have any of the biological activities which are known for the flavodoxins (Hinkson and Bulen, 1967).

**Resolution of the Shethna Flavoprotein**

The FMN chromophore is readily dissociated from the protein moiety by treatment with 3% trichloroacetic acid (TCA) (Hinkson, 1968). The precipitated protein easily dissolves in basic buffer and will rebind FMN. This technique gives rise to a variable amount of a high molecular weight protein component, as shown by gel filtration column chromatography on Sephadex G-100 (Fig. 34). This high molecular weight material was not formed when the TCA treatment was done in the dark in the presence of 10\(^{-3}\)M dithiothreitol (Fig. 34). Although no confirming experiments were carried out, it is likely that the high molecular weight material is a dimeric form of the apo-protein with an intermolecular disulfide bond linking the
Fig. 34. Sephadex G-100 Column Chromatography of the Shethna Flavoprotein and Apoprotein.

---- Flavoprotein

..... Apoprotein prepared in the dark and in the presence of $1 \times 10^{-3}$M dithiothreitol

----- Apoprotein prepared in the light without dithiothreitol

The buffer used was 0.025M phosphate, pH = 7.0 and the column dimensions were 1.8 x 50 cm
Fig. 34. Sephadex G-100 Column Chromatography of the Shethna Flavoprotein and Apoprotein.
two protein molecules. Since flavin is a good oxidizing agent in acid solution and is also a good photooxidant, the precautions taken also protect against possible oxidation of susceptible amino acids such as tryptophan, methionine, and cysteine.

The ultraviolet absorption spectrum (Fig. 35), as well as the visible spectrum, of the apoprotein indicates no residual flavin, and is similar to the general ultraviolet absorption spectra of many proteins containing aromatic amino acids. The circular dichroism spectrum (Fig. 35) shows optical activity due to transitions of the aromatic amino acids tryptophan and tyrosine. Resolution of the CD spectrum into a minimum number of Gaussian components gives five bands with four positive transitions at 297, 288, 282, and 276 nm and a negative band at 265 nm. Based on the results of Strickland et al. (1969), the CD spectrum seems to be due primarily to indole electronic transitions, although tyrosine transitions probably overlap below 285 nm. The negative indole transition at 303 nm seen in the CD spectrum of chymotrypsinogen (Strickland et al., 1969) is not present in the Shethna apoprotein spectrum (Fig. 35), even though the other dichroic bands are quite similar. Both \( l_{L_A} \) and \( l_{L_B} \) indole transitions are apparent, which, according to the Strickland analysis, suggest that the various tryptophyl residues may be both exposed and "buried" with respect to the solvent. The fluorescence
Fig. 35. Near Ultraviolet CD and Absorption Spectra of the Shethna Apoprotein.

..... Absorption spectrum
——— CD spectrum
——— Curve resolution

The spectra were measured on protein solutions in 0.025M phosphate buffer, pH = 7.0.
Fig. 35. Near Ultraviolet CD and Absorption Spectra of the Shethna Apoprotein.
emission spectrum of the tryptophan residues is also intermediate between the emission maxima expected for tryptophan in nonpolar solvents and in polar solvents (D'Anna and Tollin, 1970).

A comparison of the far ultraviolet circular dichroism spectrum of the apoprotein with that of the native flavoprotein, indicates no extensive denaturation upon flavin removal (Fig. 36). The differences seen in the CD spectra of these two species indicate a slightly different conformation for the apoprotein. Rebinding of FMN gives a complex whose far UV CD spectrum is indistinguishable from that of the native flavoprotein (Fig. 36), thereby providing evidence for a completely reversible flavin-protein recombination. Likewise, the CD and absorption spectra of the reconstituted flavoprotein in the near ultraviolet and visible region are indistinguishable from those of the native flavoprotein.

A comparison of the amide chromophore Cotton effects of the Shethna flavoprotein and apoprotein with those of model polypeptides (Beychok, 1968) suggests the presence of both alpha helix and beta structure in the secondary conformation of the polypeptide chain. The negative CD bands at 222 nm and at 208 nm are characteristic of an alpha helix. Using the values of the mean residue ellipticities at 222 nm (Fig. 36) (-7,800 deg-cm$^2$-decimole$^{-1}$ for the holoprotein and -10,000 deg-cm$^2$-decimole$^{-1}$ for the apoprotein),
Fig. 36. Far Ultraviolet CD Spectra of the Shethna Flavoprotein, Apoprotein and Reconstituted Flavoprotein.

The spectra were measured on protein solutions in 0.025M phosphate buffer, pH = 7.0. One mm pathlength quartz cells were used in all measurements.
helix contents of 26% and 33% are estimated, respectively.
A 30% helix content is estimated from the magnitude of the
233 nm trough in the optical rotatory dispersion spectrum
of the apoprotein.

Alpha-helical polypeptides have a positive CD band
at 191-192 nm, whereas polypeptides with a large amount of
beta-structure have a positive band at 195 nm (Beychok, 1968).
Thus, the occurrence of a positive CD band at 195 nm in the
spectra of the apoprotein and flavoprotein suggests a sub­
stantial amount of beta structure. The presence of beta
structure would also be expected to increase the magnitude
of the 222 nm negative band, inasmuch as a peptide chain
having that conformation has a negative CD band at 217-218 nm
(Beychok, 1968). That this is the case is shown in Figure 36,
where it can be seen that the 208 nm band is approximately
60% as intense as the 222 nm band in both the flavoprotein
and the apoprotein. In a true alpha-helical polypeptide,
the magnitude of both of these bands would be approximately
the same (Beychok, 1968). This constitutes further evidence
for the presence of substantial amounts of beta structure
in the Shethna protein and also indicates that the estimated
percentage of alpha helix is probably twice as large as is
actually present. Therefore, a helical content of approx­
imately 15% represents a more realistic value.
The Binding of Flavin Analogs to the Shethna Apoprotein

Equilibrium Binding

The equilibrium binding of flavin analogs to the Shethna apoprotein was measured by the quenching of flavin fluorescence for all analogs except lumiflavin, for which optical difference spectra were used. The binding of all analogs to the protein was in a 1:1 molar ratio, as indicated by the linearity of the binding plots (Figs. 37 and 38). As is shown in Table XI, FMN is quite strongly bound to the protein. This value is in good agreement with that published by Hinkson (1968). That the side-chain hydroxyl groups do not appreciably contribute to the binding energy is apparent from the similar $K_a$ values for FMN and deoxyFMN and for riboflavin and deoxyriboflavin (Table XI). The binding energy contributed by the terminal phosphate group is 2.8 Kcal-mole$^{-1}$ for FMN, 2.3 Kcal-mole$^{-1}$ for deoxyFMN and 1.9 Kcal-mole$^{-1}$ for isoFMN (by comparison of the $K_a$ values with the non-phosphorylated flavins). A dramatic difference is observed in the binding properties of 3-methylFMN and 3-methylriboflavin. The 3-methylFMN is bound quite strongly while 3-methylriboflavin is not bound at all. No fluorescence quenching, absorption spectral perturbations, or changes in the circular dichroism spectrum
Fig. 37. Equilibrium Binding of FMN Analogs to the Shethna Apoprotein.

The solid-line plots correspond with the values on the left-hand ordinate while the dashed-line plots correspond with the values on the right-hand ordinate.

- FMN
- Δ DeoxyFMN
- x IsoFMN
- o 3-methylFMN

All measurements were made in 0.025M phosphate buffer, pH = 7.0 at 25±2°C.
Fig. 37. Equilibrium Binding of FMN Analogs to the Shethna Apoprotein.
Fig. 38. Equilibrium Binding of Riboflavin Analogs and FAD to the Shethna Apoprotein.

- ▲ Deoxyriboflavin
- ○ N-10-carboxybutylisoalloxazine
- X Isoriboflavin
- ● Riboflavin
- ■ FAD
- △ Lumiflavin

All measurements were made in 0.025M phosphate buffer, pH = 7.0 at 25°C.
Fig. 33. Equilibrium Binding of Riboflavin Analogs and FAD to the Shethna Apoprotein.
## TABLE XI
Equilibrium Binding Constants of Flavin Analogs to the Shethna Apoprotein

<table>
<thead>
<tr>
<th>Flavin</th>
<th>$K_2$</th>
<th>Correlation Coefficient</th>
<th>$\Delta F^o$(Kcal/mole)</th>
<th>$\Delta F_u$(Kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN</td>
<td>$2.2 \times 10^8$</td>
<td>0.960</td>
<td>-11.3</td>
<td>-13.7</td>
</tr>
<tr>
<td>deoxyFMN</td>
<td>$1.3 \times 10^8$</td>
<td>0.986</td>
<td>-11.0</td>
<td>-13.4</td>
</tr>
<tr>
<td>3-methyl-FMN</td>
<td>$3.7 \times 10^7$</td>
<td>0.983</td>
<td>-10.3</td>
<td>-12.7</td>
</tr>
<tr>
<td>isoFMN</td>
<td>$4.8 \times 10^7$</td>
<td>0.905</td>
<td>-10.4</td>
<td>-12.8</td>
</tr>
<tr>
<td>riboflavin</td>
<td>$1.8 \times 10^6$</td>
<td>0.991</td>
<td>-8.5</td>
<td>-10.9</td>
</tr>
<tr>
<td>deoxy-riboflavin</td>
<td>$2.4 \times 10^6$</td>
<td>0.931</td>
<td>-8.7</td>
<td>-11.1</td>
</tr>
<tr>
<td>3-methyl-riboflavin</td>
<td>NOT BOUND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isoriboflavin</td>
<td>$1.7 \times 10^6$</td>
<td>0.912</td>
<td>-8.5</td>
<td>-10.9</td>
</tr>
<tr>
<td>FAD</td>
<td>$1.3 \times 10^6$</td>
<td>0.915</td>
<td>-8.3</td>
<td>-10.7</td>
</tr>
<tr>
<td>lumiflavin</td>
<td>$2.2 \times 10^5$</td>
<td>0.959</td>
<td>-7.2</td>
<td>-9.6</td>
</tr>
<tr>
<td>N-10-ω-carboxybutylisoalloxazine</td>
<td>$3.1 \times 10^6$</td>
<td>0.972</td>
<td>-8.8</td>
<td>-11.2</td>
</tr>
<tr>
<td>tetra-O-acetyl riboflavin</td>
<td>NOT BOUND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-thioFMN</td>
<td>NOT DETERMINED</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The association constant ($K_a$) was estimated from a least squares analysis of the binding plots (Figs. 37 and 38). The correlation coefficient is given for each plot. The standard molar free energy of binding ($\Delta F^o$) was calculated from the expression: $\Delta F^o = -RT\ln K_2$. The unitary free energy of binding ($\Delta F_u$) was calculated using the standard molar free energy less the cratic entropy value of 8 entropy units (Kauzmann, 1959).
were observed upon adding apoprotein to a solution of 3-methylriboflavin.

Modification of the isoalloxazine ring results in a decrease in binding energy of ≈1 Kcal-mole⁻¹ (by comparison of the binding energies of isoFMN and 3-methylFMN with that of FMN). The association constant for the 2-thioFMN-protein complex was not determined inasmuch as 2-thioFMN is nonfluorescent (Mitchell and Hastings, 1969). However, this flavin analog is bound quite strongly since it cannot be dialyzed away from the protein. A difference spectrum titration of 2-thioFMN with the apoprotein also indicated a large binding constant. However, the high concentration required (≈4x10⁻⁵M) made the determination of an equilibrium constant impossible.

The steric and electrostatic importance of the ribityl phosphate side chain is evident in the binding properties of FAD, N-10-α-carboxybutylisoalloxazine, lumiflavin and tetra-O-acetylriboflavin (Table XI). FAD is bound with about 3 kcal-mole⁻¹ less energy than FMN, even though a negatively charged pyrophosphate group is present. N-10-α-carboxybutylisoalloxazine is bound with about 2 Kcal-mole⁻¹ less energy than deoxyFMN, although the former compound has a negatively charged carboxyl group. Lumiflavin is bound considerably less strongly than any of the other analogs. The bulky acetyl side-chain groups on tetra-O-acetyl riboflavin prevent binding
altogether, indicating a close fit between protein and flavin side chain.

The lumiflavin binding constant indicates that the majority of the free-energy (9-10 Kcal-mole$^{-1}$) in flavin-protein binding is derived from the isoalloxazine-protein interactions (the difference in binding energy between lumiflavin and deoxyriboflavin is only 1.5 Kcal-mole$^{-1}$). Inasmuch as the free energy of association of flavin complexes with phenols or indoles is about 3 Kcal-mole$^{-1}$ (Tollin, 1968b), it seems likely that several protein residues interact with the isoalloxazine ring. The differences in CD spectra observed between the FMN and 3-methylFMN protein complexes indicates that the 3 position of the FMN molecule is probably one site of interaction. This is further substantiated by the shift in pK of the N-3 proton from ~10 in unbound FMN (Ehrenberg and Hemmerich, 1968) to above 11 for the Shethna flavoprotein (as determined by measuring the optical difference spectrum in the visible region as a function of pH). A true pK value is technically impossible to obtain due to major changes in protein structure which occur above pH = 12.0 (as evidenced by CD spectra).

The positive free energy contribution to binding from the 3-methyl group in 3-methylriboflavin must be quite large, since it overcomes the negative free energy of the flavin ring and side chain interactions with the protein.
Such a large free energy term could not be expected to be counteracted by the 2.5-3 kcal-mole\(^{-1}\) estimated for the phosphate interaction with the protein. Thus, to explain the lack of binding of 3-methylriboflavin and the strong binding of 3-methylFMN, it is necessary to postulate that the protein-flavin interactions are different with the FMN analogs than with the riboflavin analogs. A possible cause of such a different interaction could be a phosphate-induced protein change in the flavin binding site (see below for further discussion).

**Fluorescence Quenching of the Flavin Analogs upon Binding**

The flavin fluorescence is completely quenched in all of the FMN analog-protein complexes. With the riboflavin analogs, the flavin fluorescence is completely quenched only for riboflavin and isoriboflavin. For those analogs without side-chain hydroxyl groups (deoxyriboflavin, N-10-\(\omega\)-carboxybutylisoalloxazine, and lumiflavin), the ratio of fluorescence of the protein-flavin complex to that of the unbound flavin is 0.49, 0.48, and 0.88, respectively. These data suggest that the hydroxyl groups play a role in the non-radiative dissipation of singlet energy in the riboflavin analogs (see above for evidence that ring-side chain interaction is increased upon binding FMN to apoprotein) and that there is more than one mechanism for the total quenching of protein-bound flavin fluorescence (since
deoxyFMN fluorescence is totally quenched). These differences in fluorescence quenching are not due to binding energy differences, since, for example, deoxyriboflavin is bound as strongly as riboflavin (Table XI). The total quenching of the fluorescence of deoxyFMN demonstrates that quenching by protein groups is a much more efficient process with this analog than is the case with deoxyriboflavin. This is a further indication of the difference in protein-flavin interactions in the FMN analogs and the riboflavin analogs. The ratio of flavin fluorescence of the FAD-protein complex to that of free FAD is 0.34. This is difficult to compare directly with the other quenching data since FAD has only one-fifth the fluorescence of the other flavin analogs (Penzer and Radda, 1967). It is clear, however, that the adenylic acid group does interfere with the quenching processes.

**CD Spectra of Protein-bound Riboflavin Analogos**

In order to be sure that the riboflavin analogs and FAD are bound in a specific manner, the circular dichroism spectra of these protein-flavin complexes were measured (Figs. 39 and 40). The CD of the protein complexes of riboflavin, FAD, and isoriboflavin are quite similar to that of the FMN-protein complex (Fig. 8), both with respect to shape and intensity. Similarly, the CD spectra of the protein complexes of lumiflavin, deoxyriboflavin, and N-10-ω-carboxybutylisoalloxazine (Fig. 39) resemble that
Fig. 39. CD Spectra of the Complexes of the Shethna Apoprotein with Riboflavin, Isoriboflavin and FAD in the Visible Region.

----- Isoriboflavin
..... Riboflavin
----- FAD

All spectra were measured in 0.025M phosphate buffer, pH = 7.0.
Fig. 39. CD Spectra of the Complexes of the Shethna Apoprotein with Riboflavin, Isoriboflavin and FAD in the Visible Region.
Fig. 40. CD Spectra of Complexes of the Shethna Apoprotein with Those Riboflavin Analogs without Side-chain Hydroxyl Groups in Visible Region.

..... Lumiflavin
----- N-10-\(\omega\)-carboxybutylisoalloxazine
----- Deoxyriboflavin

All spectra were measured in 0.025M phosphate buffer.
Fig. 40. CD Spectra of Complexes of the Shethna Apoprotein with Those Riboflavin Analogs without Side-chain Hydroxyl Groups in Visible Region.
of the deoxyFMN-protein complex (Fig. 10). The low rotational strength of the long wavelength positive dichroic band is evident in the complexes of all three of the deoxy analogs, as was found for the deoxyFMN-protein complex. This is again evidence that the optical activity in the region reflects ring-side chain interactions. The CD spectrum of the 2-thioFMN-protein complex (Fig. 41) is much different in shape and has a greater intensity than the spectrum of the free FMN analog (which is quite similar to the CD spectrum of FMN although red-shifted).

The similar CD spectra of the bound riboflavin analogs\(^1\) to the protein-bound FMN analogs indicate no major differences in interaction (although minor differences certainly exist).\(^2\) The apoprotein had very little effect on the CD spectra of 3-methylriboflavin and tetra-O-acetylriboflavin, thereby providing further evidence that these analogs do not bind. The increase in rotational strength in the long-wavelength region for the FAD-apoprotein complex also indicates (as was discussed above) that the side-chain hydroxyl groups come into proximity to the ring upon binding.

\(^1\)The rotational strengths of these complexes are actually higher than indicated in the figure, since the spectra were taken on equimolar solutions of apoprotein and flavin and were not corrected for the actual amount of flavin bound.

\(^2\)The near ultraviolet CD spectrum (250-300 nm) of the riboflavin-apoprotein complex was also quite similar to that of the FMN-apoprotein complex.
Fig. 41. CD and Absorption Spectra of the 2-thio-FMN-Shethna Apoprotein Complex in the Visible Region.

--- Absorption spectra

--- CD spectra

Note that the absorption spectrum has been inverted to facilitate comparison. The spectra were measured on protein solutions in 0.025M phosphate—0.05M EDTA buffer, pH = 7.0.
Fig. 41. CD and Absorption Spectra of the 2-thio-FMN-Shethna Apoprotein Complex in the Visible Region.
Kinetics of Flavin Binding to the Apoprotein

Further evidence for differences in the mode of binding of the various flavin analogs can be seen in the binding kinetics. FMN binding follows second order kinetics with a rate constant of $2.0 \times 10^5 \text{M}^{-1}\text{sec}^{-1}$ (Fig. 42, and Table XII). As indicated in Figure 42, equimolar quantities of FMN and protein gave linear second order plots at three different concentrations. The 3-methylFMN and isoFMN analogs gave results which were quite similar to FMN with approximately the same rate constants (Table XII). DeoxyFMN was bound more rapidly than FMN (rate constant about three times that of FMN (Table XII)). Riboflavin and isoriboflavin were also bound more rapidly, with half-times of about 0.5 seconds, as compared to 2.5 seconds for FMN. The binding rate was too rapid to determine whether the process was first or second order.

These data clearly demonstrate the importance of the side chain in the binding process. The increase in binding rate upon removal of the terminal phosphate group indicates that the phosphate-protein interaction occurs within the protein structure and not on the surface, and that this interaction is rate-limiting. Tryptophan fluorescence quenching is also seen upon binding the flavin phosphates (D'Anna and Tollin, 1970). The second-order rate constants for tryptophan quenching are approximately the same as for
Fig. 42. Second-order Rate Plot of FMN Binding to the Shethna Apoprotein.

The rate of binding was determined flurometrically in 0.025M phosphate buffer, pH = 7.0 at ±25°C. The individual plots are for the following equimolar concentrations of FMN and apoprotein.

- 2 x 10^{-6} M; k = 2.0 x 10^{5} M^{-1} sec^{-1}
- 1 x 10^{-6} M; k = 1.8 x 10^{5} M^{-1} sec^{-1}
- 4 x 10^{-7} M; k = 2.0 x 10^{5} M^{-1} sec^{-1}
Fig. 42. Second-order Rate Plot of FMN Binding to the Shethna Apoprotein.
# TABLE XII

Second-order Rate Constants for Flavin Binding to the Shethna Apoprotein

<table>
<thead>
<tr>
<th>Flavin</th>
<th>$k(M^{-1}sec^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMN</td>
<td>$2.0 \times 10^5$</td>
</tr>
<tr>
<td>isoFMN</td>
<td>$2.9 \times 10^5$</td>
</tr>
<tr>
<td>3-methylFMN</td>
<td>$1.7 \times 10^5$</td>
</tr>
<tr>
<td>deoxyFMN</td>
<td>$5.7 \times 10^5$</td>
</tr>
<tr>
<td>riboflavin</td>
<td>$t_{1/2} = 0.5$ sec</td>
</tr>
<tr>
<td>isoriboflavin</td>
<td>$t_{1/2} = 0.5$ sec</td>
</tr>
<tr>
<td>deoxyriboflavin</td>
<td>too fast to measure</td>
</tr>
</tbody>
</table>

Note: All kinetic rate constants were determined at a concentration of $2 \times 10^{-6}$M flavin and $2 \times 10^{-5}$M protein in 0.025M phosphate buffer $pH = 7.0$ at room temperature ($25 \pm 2^\circ C$).
flavin quenching. This is further evidence of a crucial role of the phosphate group in flavin binding.

The importance of the side-chain hydroxyl groups is also qualitatively seen in a comparison of deoxyriboflavin with riboflavin (Table XII). The rate of deoxyriboflavin binding was too fast to be measured under the conditions employed. Thus, the side-chain hydroxyl groups are also involved in the rate-limiting step of the binding process, although as we have seen, they do not significantly contribute to the binding energy (Table XI). Since flavin ring modifications do not appreciably affect the binding rate, the side chain interactions with the protein represent the initial, and rate-determining, step in the binding of the flavin coenzyme.

Properties of Semiquinones of Flavoenzymes and FAD-analog Complexes of the Shethna apoprotein

Photochemical Reduction

A further difference between the riboflavin and FAD analog complexes of the Shethna apoprotein is that stable semiquinones are formed only with the FAD-protein complexes. The semiquinones can be produced either by dithionite reduction or by illumination in the presence of EDTA. The absorption and circular dichroism spectra of the semiquinone species were given in a previous section. Only fully-reduced forms were observed with the protein complexes of the riboflavin analogs or of FAD. When oxygen
is added to these reduced species, they are very rapidly oxidized all the way to the fully-oxidized forms.

The rate of photoreduction of the Shethna flavoprotein is quite slow (Table XIII), in agreement with earlier studies (Massey and Palmer, 1966; McCormick, Koster, and Veeger, 1967). The reconstituted FMN-protein complex is photoreduced at approximately the same rate, again indicating no irreversible changes upon resolution and reconstitution. The protein complexes of 3-methylFMN, deoxyFMN, and 2-thioFMN are reduced more rapidly than the FMN protein, whereas the isoFMN derivative reduced somewhat more slowly (Table XIII). The photoreducibility of the bound FMN is thus quite sensitive to the presence of side-chain hydroxyl groups and to modifications in the pyrimidine ring, but less sensitive to modification in the benzenoid portion of the flavin ring system.

A plot of $A_{450}$ vs. $A_{580}$ should be linear for a one-electron reduction of the flavoproteins, but will become nonlinear if further reduction of the flavin to the hydroquinone occurs. Figure 43 indicates that very little hydroquinone is produced in the photoreduction of the protein complexes of FMN and isoFMN, while some complete reduction occurs with the deoxyFMN, 3-methylFMN and 2-thioFMN analogs. Substantial hydroquinone formation occurs in the photoreduction of the Shethna flavoprotein in $3M \ (NH_4)_2SO_4$ (Fig. 42). Some flavin hydroquinone


TABLE XIII

Photochemical Semiquinone Production and Chemical Reduction of Flavoenzymes and FMN Analog-protein Complexes at pH = 7.0

<table>
<thead>
<tr>
<th>Flavoenzyme</th>
<th>Rate of Photoreduction $t_{1/2}$ (hrs)</th>
<th>Dithionite Stability of FH.</th>
<th>Rate of Dithionite Reduction $t_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shethna flavoprotein</td>
<td>70</td>
<td>yes</td>
<td>16 min</td>
</tr>
<tr>
<td>FMN-apoprotein</td>
<td>63</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>isoFMN-apoprotein</td>
<td>125</td>
<td>no</td>
<td>fast</td>
</tr>
<tr>
<td>deoxyFMN-apoprotein</td>
<td>5.5</td>
<td>yes</td>
<td>5 min</td>
</tr>
<tr>
<td>3-methylFMN-apoprotein</td>
<td>4.8</td>
<td>no</td>
<td>fast</td>
</tr>
<tr>
<td>2-thioFMN-apoprotein</td>
<td>3.7</td>
<td>no</td>
<td>fast</td>
</tr>
<tr>
<td>R. rubrum flavodoxin</td>
<td>28.8</td>
<td>yes</td>
<td>-</td>
</tr>
<tr>
<td>P. elsdenii flavodoxin</td>
<td>1.5</td>
<td>no</td>
<td>-</td>
</tr>
<tr>
<td>C. pasteurianum flavodoxin</td>
<td>0.4</td>
<td>no</td>
<td>-</td>
</tr>
<tr>
<td>Shethna flavoprotein in 3M (NH$_4$)$_2$SO$_4$</td>
<td>0.3</td>
<td>no</td>
<td>fast</td>
</tr>
<tr>
<td>Shethna flavoprotein + 5% M/MM FMN</td>
<td>5.0</td>
<td>yes</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 43. Relation between Decrease of Oxidized Flavoprotein Absorbance and Increase in Semiquinone Absorbance during Photo-reduction.

All photoreductions were carried out 4°C in 0.025M phosphate--0.05M EDTA buffer, pH = 7.0. Further details are given in the experimental section.

- Shethna flavoprotein (native and reconstituted)
- Shethna flavoprotein plus 3M (NH₄)₂SO₄

The FMN analog-Shethna apoprotein complexes are:

- DeoxyFMN
- 3-methylFMN
- IsoFMN
- 2-thioFMN (for this analog the ordinate is the absorbance at 490 nm and the abscissa the absorbance at 670 nm)
Fig. 43. Relation between Decrease of Oxidized Flavoprotein Absorbance and Increase in Semiquinone Absorbance during Photo-reduction.
formation was also found to occur with *P. elsdenii* and *C. pasteurianum* flavodoxins but not with *R. rubrum* flavodoxin.

*R. rubrum* flavodoxin is photoreduced at an intermediate rate, while the *P. elsdenii* and *C. pasteurianum* flavodoxins are photoreduced much more rapidly than any of the other species (Table XIII). The rate of photoreduction of the Shethna flavoprotein is increased quite dramatically by high salt concentration (3M (NH₄)₂SO₄) or by the addition of free flavin (Table XIII). The enhanced rate and increased formation of flavohydroquinone in the presence of high salt concentration is probably due partly to the presence of free flavin dissociated from the protein and also to changes in protein structure. The latter is suggested by the lack of semiquinone stability to dithionite reduction under these conditions (see below). The CD spectra of the oxidized and semiquinone forms of the Shethna flavoprotein in 3M (NH₄)₂SO₄ were identical in the visible and the near ultraviolet spectral regions to those of the flavoprotein in low ionic strength buffer (0.025M phosphate - 0.05M EDTA). Thus, any protein structural changes were not reflected in the CD data and, consequently, are probably fairly localized.

An enhanced rate of photoreduction of D-amino acid oxidase upon adding free FMN or FAD has also been observed (McCormick *et al.*, 1967). Such effects are probably due to the rapid reduction of free flavin by illumination, followed
by reduction of the protein-bound flavin by free flavin hydroquinone.

The absorption and CD spectra of the 2-thioFMN-Shethna protein semiquinone are presented in Figure 44. The long wavelength absorption band (670 nm) indicates that this semiquinone is also in its protonated form. The photo-reduced material was paramagnetic (as were all of the semiquinones of the other flavin analogs). The CD spectrum (Fig. 44) shows no optical activity in the long wavelength (600 nm) region, suggesting weak side-chain ring interaction (see above). The other bands in the absorption spectrum have their counterparts in the CD spectrum. The CD and absorption spectra of the reoxidized material were indistinguishable from that of the non-irradiated 2-thio-FMN-protein.

Chemical Reduction by Dithionite

At neutral pH and at low ionic strength, the semiquinone form of the Shethna flavoprotein cannot be further reduced even with a large excess of dithionite (Hinkson and Bulen, 1967). This reconstituted FMN-protein complex also retains this property (Hinkson, 1968). Our results (Table XIII) verify these observations. Also of interest is that ring-modified FMN analog-protein complexes are reduced to the hydroquinone form, whereas removal of the hydroxyl groups (deoxyFMN) has no effect on semiquinone stability to dithionite. In the latter case, the rate of
Fig. 44. CD and Absorption Spectra of the Semiquinone Form of the 2-thioFMN-Shethna Apoprotein Complex in the Visible Region.

The spectra were measured on protein solutions in 0.025M phosphate--0.05M EDTA buffer, pH = 7.0. Note that the absorption spectrum is inverted to facilitate comparison.
Fig. 44. CD and Absorption Spectra of the Semiquinone Form of the 2-thioFMN-Shethna Apoprotein Complex in the Visible Region.
reduction of the oxidized form to the semiquinone is increased to about two and half times that of the FMN-protein complex (Table XIII, and Fig. 45). All of the riboflavin analog-protein complexes were reduced to the hydroquinone form, thus again indicating the importance of the terminal phosphate group in determining protein-bound flavin properties.

*R. rubrum* flavodoxin is also resistant to reduction past the semiquinone form by dithionite (Cusanovich, 1970), whereas *P. elsdenii* and *C. pasteurianum* flavodoxins are reduced to the hydroquinone form (Mayhew et al., 1969; Knight and Hardy, 1967). CD spectral properties which correlate with these chemical properties have been discussed previously (see above).

High salt concentration (3M $\text{NH}_4\text{SO}_4$) removes the semiquinone stability of the Shethna flavoprotein to dithionite reduction, while added free flavin has no effect (Table XIII). The addition of a catalytic amount of methyl viologen (1 : 100 M/M) increases the rate of reduction by a factor of 80 (Fig. 45), but has no effect on semiquinone stability to further reduction. The reaction rate also follows pseudo-first-order kinetics (Fig. 45).

At pH = 8.0, the Shethna flavoprotein semiquinone is no longer resistant to further reduction. The semiquinone form is, however, still an intermediate in the reduction to the hydroquinone form, as is shown in Figure 46. Thus, one
Fig. 45. First-order Rate Plot of Flavoprotein Reduction by Dithionite.

All reactions were carried out in 0.025M phosphate buffer, pH = 7.0 at room temperature. The reductions were carried out under anaerobic conditions with a large excess (≈1mg) of dithionite.

- Shethna flavoprotein
- Shethna flavoprotein plus a catalytic amount of methyl viologen (1/100 l/l)
- DeoxyFlav-Shethna apoprotein complex
Fig. 46. Rate of Shethna Flavoprotein Reduction by Dithionite as a Function of pH.

The reactions were carried out in 0.1M Tris buffer at the appropriate pH under anaerobic conditions with an excess (~1mg) of dithionite at 25 2°C.

The solid lines refer to the change in absorbance at 452 nm while the dashed lines designate the change in absorbance at 580 nm. The absorption changes at both wavelengths were monitored during the same reaction.

- pH = 7.0
- pH = 7.7
- pH = 10.4
Fig. 46. Rate of Shethna Flavoprotein Reduction by Dithionite as a Function of pH.
observes a steady-state in semiquinone concentration with a
time course consistent with its being an intermediate
species.

At pH = 7.0, the absorption changes at 452 nm and
at 580 nm follow a monophasic (exponential) curve, while at
pH's between 7.0 and 8.0, the change in absorbance at
452 nm is initially slow and then decreases more rapidly at
later times (Fig. 46). Above pH = 8.0, monophasic kinetics
are again followed. This type of behavior is indicative of
an auto-catalytic reduction of the oxidized flavoprotein to
the hydroquinone form via the semiquinone. The latter can
be generated by a disproportionation reaction and, according
to this scheme, reacts more rapidly with dithionite than does
the oxidized form.

\[
\begin{align*}
1. \text{PF} & \quad \text{S}_2\text{O}_4^2\text{−} \quad \text{PFH}^• \quad \text{S}_2\text{O}_4^2\text{−} \quad \text{PFH}_2 \\
\text{slow} & \quad \text{fast} & \\
2. \text{PF} + \text{PFH}_2 & \quad \text{fast} \quad 2\text{PFH}^•
\end{align*}
\]

The addition of flavoprotein in the hydroquinone form to
oxidized material results in a very rapid production of
semiquinone (less than 5 seconds), thereby establishing that
reaction 2 could be operative. Upon adding oxygen to the
flavoprotein hydroquinone form, a rapid oxidation to the
semiquinone form results at all pH values examined. Thus,
no change in semiquinone stability to oxygen occurs in going
from pH 7.0 to pH 8.0 (see below). These results are in
agreement with the proposed pathway of oxygen oxidation of the dehydrogenase hydroquinones by Massey et al. (1969a). (See Introduction.)

The pH dependence of hydroquinone formation by an excess of dithionite was investigated. The reaction was allowed to proceed for a long enough time to assure that equilibrium had been achieved. The fact that this is an equilibrium process can only be explained if the species which is ionizing is PFH₂. If either PF or PFH⁻ were ionizing, the excess dithionite would serve to drive the reaction to completion. If one assumes a one-proton process, a plot of the ratio of PFH₂ to PFH⁻ vs. 1/(H⁺) should be linear. As is shown in Figure 47, this is the case. Note that the plot goes through the origin. This is consistent with an ionization of the hydroquinone species rather than the semiquinone or oxidized forms. No differences were caused by the presence or absence of a catalytic amount of methyl viologen, although the equilibrium was established much faster when the viologen was present. The slope of the plot gives an apparent pK for the ionization of 7.0.

These results indicate that the removal of a proton from either a protein group or the flavin molecule in the hydroquinone form of the protein is responsible for the loss of stability of the flavin semiquinone to dithionite reduction. The identical behavior with and without methyl
The semiquinone (PFH*) concentration was estimated from the absorbance at 580 nm at equilibrium. The hydroquinone (PFH) was assumed to account for the rest of the total flavin concentration. The buffers used were 0.1M Tris—0.33M phosphate above pH 7.0 and 0.1M phosphate below pH = 7.0. No differences were observed with the different buffers at the same pH. The (●) denote the presence of a catalytic amount of methyl viologen and the (x) are samples with no methyl viologen. The temperature was 25±1°C. A large excess of dithionite (~1mg) was added to the flavoprotein under anaerobic conditions. The pH was measured before and after the measurement.
Fig. 47. Ratio of Shethna Flavoprotein Hydroquinone to Semiquinone as a Function of the Reciprocal Hydrogen Ion Concentration.
viologen establishes that any changes in dithionite reducing potential at different pH values are not important. Also any redox potential changes which do occur (Munemori, 1958) would not effect the reduction since a large excess (~50-fold M/M) of dithionite was used. The only protein group which normally ionizes in this pH region is the imidazole ring. However, the Shethna flavoprotein does not contain histidine (see amino acid analysis data), and thus a perturbed carboxyl or amino group would have to be considered. The enormous shift in pK which would have to occur for either of these groups to ionize at pH = 7.0 makes this possibility unlikely, although still feasible.

A more likely candidate is the ionization of the FMN terminal phosphate group. The second ionization of phosphoric acid is at pH = 7.2. Removal of a proton could disrupt the phosphate-protein bond thereby changing the environment of the bound flavin so as to allow formation of the hydroquinone form.\textsuperscript{1} In this respect, protein-bound riboflavin is completely reduced by dithionite (see above).

A third possibility which can account for these data is the ionization of the neutral flavin hydroquinone to the anion form. Free flavin hydroquinone ionizes with a pK = 6.2 (Ehrenberg and Hemmerich, 1966). Since flavin hydroquinone exists in a bent "butterfly" configuration

\textsuperscript{1}It is also possible that the phosphate-binding group on the protein is involved.
(see Introduction), steric constraints in the flavin binding site may allow only the planar oxidized and semiquinone flavin rings to exist in the Shethna flavoprotein. Ionization of the flavin hydroquinone may bring about changes in the flavin binding site (induced, for example, by changes in solvation at N-1) which could permit the bent configuration to exist. The effect of high salt (e.g., 3M \( \text{NH}_4\text{SO}_4 \)) would be expected to shift the apparent pK of 7.0 towards the true pK of 6.2. This could explain the effect of high salt concentrations in eliminating the stability of the semiquinone towards dithionite reduction. The modifications in the flavin ring (2-thio, 3-methyl and isoFMN analogs) might also be expected to influence the apparent pK of the hydroquinone form either directly (possible only with the 2-thio and isoFMN analogs) or by modifying the protein-flavin interactions. Such changes in flavin structure would not be expected to affect the phosphate ionization. Thus, of the three possibilities, the last-mentioned seems the most attractive.

**Oxidation by Molecular Oxygen**

The semiquinone form of the Shethna flavoprotein is unique because of its low reactivity towards oxygen. As indicated in Figure 4B and Table XIV, the half-time for the

1 This is very likely the case whatever the ionizing group is.
Fig. 48. First-order Rate Plots of Shethna Flavoprotein Semiquinone Oxidation by Oxygen.

Photoreduced PFH⁺ in 0.025M phosphate--0.5M EDTA pH = 7.0.

▲ 1.14 x 10⁻⁴M
● 1.46 x 10⁻⁵M
○ Dithionite-reduced PFH⁺ (5 x 10⁻⁵M) in 0.025M phosphate--0.05M EDTA buffer, pH = 7.0

x PFH⁺ photoreduced in 3M (NH₄)₂SO₄ .025M phosphate--
0.05M EDTA pH = 7.0. (The semiquinone was freed from
salts by anaerobic Sephadex G-10 column chromatography
The buffer present during the kinetic run was 0.025M
phosphate, pH = 7.0)
Fig. 43. First-order Rate Plots of Shethna Flavoprotein Semiquinone Oxidation by Oxygen.
### TABLE XIV

**Oxygen Oxidation of the Neutral Semiquinone Forms of Flavoenzymes and FMN Analog-Shethna Apoprotein Complexes**

<table>
<thead>
<tr>
<th>Flavoenzyme</th>
<th>$t_{1/2}$ (min)</th>
<th>$k$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shethna flavoprotein</td>
<td>2000</td>
<td>$3.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>Shethna flavoprotein no EDTA</td>
<td>3000</td>
<td>$2.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Shethna flavoprotein in $3M$ $(NH_4)_2SO_4$</td>
<td>41</td>
<td>$1.7 \times 10^{-2}$</td>
</tr>
<tr>
<td>FMN-apoprotein</td>
<td>2000</td>
<td>$3.5 \times 10^{-4}$</td>
</tr>
<tr>
<td>deoxyFMN-apoprotein</td>
<td>78</td>
<td>$9.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>2-thioFMN-apoprotein</td>
<td>41</td>
<td>$1.7 \times 10^{-2}$</td>
</tr>
<tr>
<td>3-methylFMN-apoprotein</td>
<td>108</td>
<td>$6.4 \times 10^{-3}$</td>
</tr>
<tr>
<td>isoFMN-apoprotein</td>
<td>44</td>
<td>$1.6 \times 10^{-2}$</td>
</tr>
<tr>
<td><em>C. pasteurianum</em> flavodoxin</td>
<td>115</td>
<td>$6.0 \times 10^{-3}$</td>
</tr>
<tr>
<td><em>P. elsdenii</em> flavodoxin</td>
<td>80</td>
<td>$8.7 \times 10^{-3}$</td>
</tr>
<tr>
<td><em>R. rubrum</em> flavodoxin</td>
<td>260</td>
<td>$2.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>glucose oxidase</td>
<td>1.2</td>
<td>0.58</td>
</tr>
</tbody>
</table>

**Note:** The rates of oxidation were determined at room temperature in 0.025M phosphate - 0.05M EDTA pH = 7.0 except where otherwise indicated. The rate of oxidation of the glucose oxidase semiquinone was determined in 0.1M acetate - 0.05M EDTA pH = 6.0. Saturating conditions of oxygen ($=10^{-3}$M) were maintained by bubbling air through the solutions at intervals. Semiquinone concentrations were 5-8 x $10^{-5}$M in all cases.
oxidation is several thousand minutes. Semiquinone forms prepared either by photoreduction or by dithionite reduction are oxidized at the same rate. However, the presence of EDTA was found to cause a fast initial phase in the oxidation which is independent of semiquinone concentration (Fig. 48). When the semiquinone was prepared by photoreduction in the presence of 2M (NH₄)₂SO₄ and 0.05M EDTA, and then freed from the salts by anaerobic Sephadex G-10 column chromatography, no initial fast phase was observed. Subsequent addition of EDTA to this material greatly increased its rate of oxidation. The dithionite-prepared semiquinone also showed an initial fast phase in the rate of oxidation (Fig. 48). These results indicate that the faster oxidation rate is caused by EDTA and not by mechanistic complexities in the pseudo-first-order oxidation of the semiquinone by oxygen, or by irreversible protein changes upon photoreduction. It is possible that EDTA-trace metal complexes could act as catalysts in the initial oxidation and become ineffective at a later stage due to poisoning.

A faster initial rate of oxidation in the presence of EDTA was also observed with the semiquinones of the 3-methylFMN-apoprotein complex, the Shethna flavoprotein semiquinone in 3M (NH₄)₂SO₄, glucose oxidase and the deoxyFMN-apoprotein complex. None of the other semiquinone species showed this behavior. In Table XIV, the rates of
oxidation given are for the slow component of the rate curve. All rates followed pseudo-first order kinetics beyond the initial stage.

The semiquinone form of the reconstituted Shethna flavoprotein was oxidized at the same rate as the native form (Table XIV), thereby providing further evidence that no irreversible changes occurred upon resolution. The reactivity of the protein-bound FMN semiquinone with oxygen is quite sensitive to modification of the isoalloxazine ring, as well as to the absence of side-chain hydroxyl groups (Table XIV). The 2-thioFMN and isoFMN semiquinones were oxidized at a rate 50 times that of FMN, while the 3-methylFMN analog was oxidized 20 times faster. The absence of ribityl hydroxyl groups (deoxyFMN) increased the rate of oxidation by a factor of 25. The presence of high salt concentration also increases the oxidation rate of the Shethna flavoprotein (a factor of 50) thereby establishing that the interactions which stabilize the semiquinone to oxygen oxidation are sensitive to ionic strength.

The semiquinone forms of P. elsdenii and C. pasteurianum flavodoxin are oxidized about 20-25 times faster than the Shethna flavoprotein (Table XIV). The pseudo-first-order rate constant for P. elsdenii flavodoxin is in good agreement with a value determined by Mayhew et al. (1969) of $8 \times 10^{-3}\text{min}^{-1}$ at pH = 5.2.
The neutral semiquinone form of glucose oxidase is oxidized at a rate which is 10 to 1000 times faster than that of the other flavoprotein semiquinones (Table XIV). This large increase in rate could be due to the presence of a small amount of the anionic radical in equilibrium with the neutral radical. Flash photolysis work has established that the unbound anionic flavin radical reacts with oxygen at least $10^5$ times faster than the neutral radical, with a second order rate constant of $5.5 \times 10^8 \text{M}^{-1}\text{sec}^{-1}$ (Vaish and Tollin, 1970). Since the pK of the glucose oxidase semiquinone is 7.5 (Massey and Palmer, 1966), at pH = 6.0 some of the anionic radical would be present. The possibility that this faster rate of oxidation is due to the anionic radical precludes a direct comparison of glucose oxidase semiquinone reactivity towards oxygen with that of the other flavoenzyme semiquinones. No spectral evidence for the presence of anionic semiquinone has been obtained for P. elsenii flavodoxin (Mayhew and Massey, 1969) or for the Shethna flavoprotein in this study, even at pH = 11. This indicates that for these two dehydrogenases, the pK of the flavin semiquinone is shifted from 8.4 to a value greater than 11.0. The differences in oxygen reactivities which we have observed for these proteins therefore cannot be due to small concentrations of anion radical in equilibrium with the neutral form.
It is of considerable interest that the redox properties of the semiquinone of *R. rubrum* flavodoxin are in many respects intermediate between those of the Shethn protein and the other flavodoxins, in view of the similar relationship which exists in the CD spectra (see above). This provides a further indication that CD properties are a sensitive indicator of flavin environment and hence redox behavior.

**Near Ultraviolet Spectral Studies of Protein-bound Flavins**

**Near UV Absorption Spectra of Oxidized and Semiquinone Flavoproteins**

In an attempt to correlate the oxygen reactivity of the flavoprotein semiquinones with structural parameters, the ultraviolet absorption and circular dichroism spectra of the oxidized and semiquinone forms were studied. The ultraviolet difference spectrum (semiquinone minus oxidized form) of the Shethna flavoprotein shows a negative band at 287 nm and a positive band at 272 nm (Fig. 49). The extinction differences are much too large to ascribe to an aromatic amino acid perturbation.

Oxidized free FMN has an absorption band at 267 nm with a molar extinction of about 32,000 M\(^{-1}\)cm\(^{-1}\) (Fig. 50). The ultraviolet spectrum of protein-bound FMN (Fig. 50) shows a hypochromic effect at this wavelength of about 20%, with little or no wavelength shift. A hyperchromic effect
Fig. 49. Resolved Ultraviolet Difference Spectra of Flavoenzyme Semiquinone Minus Oxidized Forms

The spectra were measured on protein solutions in 0.025M phosphate—0.05M EDTA buffer, pH = 7.0.
Fig. 50. Ultraviolet Spectra of Protein-bound Free FMN Analogs.

The spectra of the protein-bound flavins were measured as difference spectra of protein-flavin complex minus apoprotein in 0.025M phosphate buffer, pH = 7.0. In both cases, flavin concentration was $2.0 \times 10^{-5}$M.
is observed at longer wavelengths (280-300 nm). From the absorption spectrum of unbound, neutral, flavin semiquinone (Land and Swallow, 1969) (see Fig. 1) one would expect an ultraviolet absorption band in this species to be below 260 nm. On the basis of these observations, it is possible to ascribe the negative band in the 280 nm region of the difference spectrum (Fig. 49) to the change in absorption spectrum of the flavin in going from oxidized to the semiquinone species. The increase in extinction at 272 nm in the difference spectrum could be due to a red-shift of the UV absorption band of the semiquinone by at least 10-15 nm in going from water to the protein environment. If this is the correct explanation, one can infer that either the protein environment of the semiquinone form is quite different from that of the oxidized flavin for which no major spectral shifts are evident (Fig. 50), or that the semiquinone spectrum is more sensitive to environmental changes than is the spectrum of the oxidized form. Another alternative is to ascribe this increased absorption to a charge-transfer type of interaction between the semiquinone and an aromatic amino acid residue. The actual assignment of this increased extinction must await further experimentation.

The same difference spectral pattern is also present in all of the flavoenzyme and FAD-analog—Shethna apoprotein complexes studied (Figs. 49 and 51). The 2-thioFAD analog
Fig. 51. Resolved Ultraviolet Difference Spectra of Semiquinone FMN Analog–Shethna Apoprotein Complexes Minus Oxidized Forms.

The spectra were measured on protein solutions in 0.025M phosphate–0.05M EDTA buffer, pH = 7.0.
also has a similar difference spectrum although it is red-shifted to 318 nm (Fig. 51). In the case of the 3-methylFMN analog, the increased extinction of the oxidized flavin (Fig. 50) cancels out the positive extinction of the semiquinone form at 275 nm (Fig. 51).

In order to obtain a more accurate value for the positive extinction with each of the semiquinone species, the difference spectra were resolved into Gaussian components. This procedure minimizes cancelling effects by the negative absorption difference, as well as additive effects caused by adjacent absorption bands, as is evidently occurring with the P. elsdenii flavodoxin (Fig. 46). More error is involved in fitting the 3-methyl-FMN spectrum than in those cases in which the difference spectra have a shape similar to that of the FMN protein. A minimal error of 10% is assumed, as judged by the range of successful alternatives observed in fitting the spectra. The analyses obtained are shown by the dashed curves in Figures 49 and 51.

As is indicated in Figure 52, a relatively linear relationship exists between the extinction of the positive band in the difference spectrum and the negative logarithm of the rate of oxygen oxidation of the semiquinone form of the flavoenzyme or FMN analog-Shethna apoprotein complex. This suggests that the interactions which cause this increase in extinction also increase the activation energy for the reaction with molecular oxygen.
Fig. 52. Relation between Semiquinone Absorptivity in the UV and Oxygen Reactivity.

$\Delta \varepsilon$ is the positive increase in semiquinone extinction of the most prominent resolved band in the difference spectra (Figs. 49 and 51). The abscissa is the negative logarithm of the pseudo-first-order rate constant for flavoenzyme semiquinone oxidation by oxygen (Table XIV).
Near UV CD Spectra of Oxidized and Semiquinone Flavoproteins

The circular dichroism properties of the Shethna flavoprotein in its oxidized and semiquinone forms also reflect the patterns seen in the optical difference spectrum (Fig. 53). The negative Cotton effect at 270 nm in the oxidized form is increased by about 40% upon semiquinone formation. Also, an increase in positive dichroism is apparent at 285 nm. The semiquinone form of isoFMN shows only a slightly increased negative 270 nm CD band, while this band is decreased in rotational strength in the case of the 3-methylFMN semiquinone form (Fig. 53). The deoxyFMN-protein complex parallels the FMN-protein complex quite closely in CD spectral properties (Fig. 54).

The rotational strengths of the CD bands in the FMN analog complexes between 260 and 300 nm are significantly increased when compared to these bands in the apoprotein (Fig. 53). That this is probably due mostly to flavin transitions rather than to perturbation of the tryptophan bands is indicated by the CD spectrum of the oxidized and semiquinone forms of the 2-thioFMN-Shethna apoprotein complex (Fig. 55). The sulfur group red shifts the flavin transitions so that the tryptophyl CD bands can be observed without overlay. No appreciable differences.}

1The changes which are observed in the 235-310 nm region are considerably smaller than those which are seen with the other FMN analogs. The differences which appear between 270 and 280 nm might be due to tyrosine perturbations.
Fig. 53. Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of FMN, 3-methylFMN and Iso-FMN-Shethna Apoprotein Complexes.

The spectra were measured on protein solutions in 0.025M phosphate—0.05M EDTA buffer, pH = 7.0.
Fig. 54. Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of the DeoxyFMN-Shethna Apoprotein Complex.

The spectra were measured on protein solutions in 0.025M phosphate—0.05M EDTA, pH = 7.0.

- Oxidized form
- - - - - - - Semiquinone form
Fig. 55. Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of the 2-thioFMN-Shethna Apoprotein Complex.

The buffer for all three spectra was 0.025M phosphate--0.05M EDTA, pH = 7.0.

- - - - - Semiquinone form
- - - - - Oxidized form
- - - - - Apoprotein
in rotational strength are observed in the tryptophan CD bands of the 2-thioFAD-Shethna apoprotein complex in either the oxidized or semiquinone forms when compared to the CD properties of the apoprotein. Thus, there is no evidence from CD data for a tryptophan-flavin interaction, although the possibility does exist that optically-inactive tryptophans might interact with the flavin.

The near ultraviolet CD spectra of the \emph{P. elsdenii} and \emph{C. pasteurianum} flavodoxins are similar to the Shethna flavoprotein, although in these cases much smaller changes in rotational strength are observed at 270 nm upon semiquinone formation (Figs. 56 and 57). The CD spectral properties of \emph{R. rubrum} flavodoxin, however, are similar to those of the Shethna flavoprotein (Fig. 58). The positive CD band in this protein at 290 nm lacks the resolution seen in the Shethna flavoprotein. This could mean that there is little optical activity arising from tryptophyl residues or that these residues are in a more polar environment than in the other flavoproteins.

Although the CD spectra of the flavoenzymes and FADN analog-Shethna apoprotein complexes in their oxidized and semiquinone forms reflect to some extent the absorption spectral properties, no correlation could be made with the oxygen reactivity of the semiquinone form. This is probably due to the complex overlapping of optically active transitions both from the protein and from the flavin.
Fig. 56. Ultraviolet Circular Dichroism Spectra of *P. elsdenii* Flavodoxin in the Oxidized and Semiquinone Forms.

The buffer used was 0.025M phosphate--0.05M EDTA, pH = 7.0 for both spectral measurements.

--- Oxidized form
--- Semiquinone form
Fig. 57. Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of \textit{C. pasteurianum} Flavodoxin.

The buffer used was 0.025M phosphate--0.05M EDTA, pH = 7.0.

- Oxidized form
- Semiquinone form
Fig. 58. Ultraviolet Circular Dichroism Spectra of the Oxidized and Semiquinone Forms of *R. rubrum* Flavodoxin.

The spectra were measured in 0.025M phosphate--0.05M EDTA, pH = 7.0.

--- Oxidized form

--- Semiquinone form
Effect of pH on Semiquinone Oxidation by Oxygen

Although the semiquinone form of the Shethna flavoprotein is air-oxidized very slowly at neutral pH, this stability is considerably decreased upon increasing the pH. Above pH = 10, the flavin radical is very rapidly oxidized by oxygen (Fig. 59). A plot of the reciprocal of the apparent first order rate constant vs. $\sqrt{pH}$ is linear (Fig. 60). This suggests a one proton ionization event which results in an increase in radical reactivity towards oxygen. This ionization could involve a group in the protein responsible for semiquinone stability or the ionization of the neutral radical to the anionic form, which is known to react much more rapidly with oxygen (see above).

The possibility of semiquinone disproportionation to the fully-reduced form (which would react quickly with oxygen) is eliminated since at pH = 11.0 the half-time of disproportionation is 4.7 min (second-order rate constant of $5 \times 10^3 M^{-1}\text{min}^{-1}$) while the rate of oxidation has a half-time of 0.57 min (pseudo-first-order rate constant of 1.2 min$^{-1}$) (Fig. 61). This lack of agreement between the rate of disproportionation and the rate of oxygen oxidation is observed at other pH values and has also been seen with the semiquinone form of \textit{P. elsdenii} flavodoxin (Mayhew \textit{et al.}, 1969).
Fig. 59. The Influence of pH on the Apparent First-order Rate Constant for Oxidation of the Shethna Flavoprotein Semiquinone by Oxygen.

The buffer was 0.1M glycine. Microliter quantities of semiquinone flavoprotein were added to air-saturated buffer at the appropriate pH. The pH values were determined after the rate measurement.
Fig 59. The influence of pH on the Apparent First-order Rate Constant for Oxidation of the Shethna Flavoprotein Semiquinone by Oxygen.
Fig. 60. The Relation between the Reciprocal of the Apparent First-order Rate Constant for Semiquinone Oxidation by Oxygen and Hydrogen Ion Concentration.

The data are taken from Figure 59. $1/k$ is expressed in minutes.
Fig. 61. A Comparison of the Rate of Shethna Flavoprotein Semiquinone Disproportionation and Oxygen Oxidation at pH = 11.0.

The buffer used in both measurements was 0.1M glycine.
Fig. 61. A Comparison of the Rate of Shethna Flavoprotein Semiquinone Disproportionation and Oxygen Oxidation at pH = 11.0.
If the ionization of a protein group resulted in the increase in oxidation rate, a tyrosyl residue would be suspect, inasmuch as the pK for the ionization of a phenolic proton can occur in this pH region. The tyrosyl ionization properties of the Shethna flavoprotein, however, indicate little difference between the oxidized and semiquinone forms (Fig. 62). This suggests that tyrosyl ionization is not involved in increasing the rate of semiquinone oxidation by oxygen. The possibility of the involvement of another protein group, such as a perturbed guanidino group of arginine, cannot be entirely eliminated. However, it would seem that the more plausible explanation of this effect is the ionization of the neutral semiquinone to the anionic form. The semiquinone form of *P. elsdenii* flavodoxin also reacts more rapidly with oxygen as the pH is increased (Mayhew *et al.*, 1969). With this protein, the rate increase occurs about one pH unit lower than in the case of the Shethna flavoprotein. This would imply that the pK for the semiquinone ionization is also about one pH unit lower for *P. elsdenii* flavodoxin than for the Shethna flavoprotein. This is not unreasonable in view of the fact that the reactivity of the flavodoxin semiquinone is considerably greater than that of the Shethna protein semiquinone (see above), suggesting less effective stabilization of the neutral radical.
Fig. 62. Tyrosyl Ionization in the Oxidized and Semiquinone Forms of the Shethna Flavoprotein and in the Shethna Apoprotein.

The buffer used was 0.1M glycine.

- Apoprotein
- Semiquinone form
- Oxidized form
From the values of the slope and intercept of the plot in Figure 60, it is in principle possible to evaluate the ionization and rate constants for the processes involved in radical oxidation. However, because of the high pK and the difficulty of obtaining rate measurements at high pH's, it is impossible to get an accurate value for the intercept. If we assume a pK of approximately 11.5 (Fig. 59), the rate constant (evaluated from the slope of the line in Fig. 60) for the oxidation is $3.0 \times 10^4 \text{M}^{-1}\text{sec}^{-1}$. This can be compared with a value of $5.5 \times 10^3 \text{M}^{-1}\text{sec}^{-1}$ obtained for the reaction of the lumiflavin anion radical with O$_2$ by flash photolysis (Vaish and Tollin, 1970). Thus, if the reason for the increase in oxidation rate of the Shethna protein semiquinone as the pH is increased is that the neutral radical becomes converted to the anion radical, then it is possible to conclude that the protein-bound species is approximately $10^5$ times less reactive towards O$_2$ than is the free anion radical. Using the apparent rate constant for glucose oxidase semiquinone oxidation by oxygen (Table IV) and a pK value of 7.5 for radical ionization (Massey and Palmer, 1966), a rate constant of $3.3 \times 10^2 \text{M}^{-1}\text{sec}^{-1}$ is calculated. This value is not unreasonable when compared with the constant calculated for the Shethna flavoprotein, in view of the different pH's employed for the rate measurements.
All five tyrosyl residues are reversibly titrated in the Shethna apoprotein (Fig. 62). The difference in tyrosyl ionization between the apoprotein and the holo-protein reflects a difference in tyrosine environment upon FMN binding. This change in environment could be due to a protein conformational change caused by binding of the coenzyme (which is suggested by the far ultraviolet CD spectra, Fig. 35) or to a direct FMN-tyrosine interaction. Inasmuch as three tyrosines are not titrated at pH = 12.0 in the holoprotein, possibly both explanations are operative, since it would be difficult to envision the interaction of three tyrosines with one flavin molecule. That some direct interaction does occur between flavin and an aromatic residue is indicated by the long wavelength "tailing" and hypochromism of the absorption spectrum discussed earlier.

**The Relation between Flavin Redox Form and Protein Conformation in the Shethna Flavoprotein**

**Circular Dichroism**

Far ultraviolet circular dichroism spectra are sensitive to alterations in protein conformation which are reflected in the environment of the amide bond. No change in rotational strength or shape is observed in the CD spectrum from 240 to 190 nm between the semiquinone form and the oxidized form of the Shethna protein at pH = 7.0.
At pH = 8.0, the spectra are the same as at pH = 7.0. However, as seen above, the addition of dithionite at pH = 8.0 reduces the flavin to the hydroquinone form. The CD intensity at 222 nm of this form of the protein is also identical to that obtained with the oxidized and semiquinone forms. Due to the absorption of dithionite and its degradation products, the spectrum in this case could not be extended below 210 nm. The results of these measurements indicate no major protein conformational changes upon flavin reduction to either the semiquinone or hydroquinone forms.

Proton Magnetic Resonance

The 220 MHz proton magnetic resonance (PMR) spectra of the oxidized and semiquinone forms also indicate no major protein conformational changes (Fig. 63). The spectra also suggest that the semiquinone prepared by light reduction is identical to that prepared by dithionite reduction. This is in agreement with CD and redox properties. The proton resonances at 0 to -1 ppm are broadened upon flavin semiquinone formation (Fig. 62). PMR signals in this region have been assigned to methyl groups of amino acids such as leucine, isoleucine, etc. (McDonald and Phillips, 1967). The peaks observed in the Shethna flavoprotein are shifted upfield by 1-2 ppm relative to other proteins such as

---

1The author is grateful to Drs. W. D. Phillips and J. Glickson of E. I. DuPont de Nemours Company for providing the PMR spectra and for their assistance in the interpretation of the data.
Fig. 63. The Effect of Redox Form on the 220 MHz Proton Magnetic Resonance Spectrum of the Shethna Flavoprotein.
trypsin or pepsin. This could be due to shielding by the flavin ring. Since these amino acids are non-polar in character, these results suggest that the flavin is in a non-polar environment. (This is also suggested by the resolution of the 450 nm flavin absorption band.) The broadening of the methyl resonances by the paramagnetic flavin radical (Fig. 63) also provides evidence that the flavin is in a non-polar environment. The proton resonance at 7-7.5 ppm is in a region unique to aromatic protons (McDonald and Phillips, 1967). The sharpening of this portion of the spectrum upon flavin semiquinone formation could be due to broadening of the proton resonances of an aromatic residue in proximity to the flavin, thereby eliminating the overlapping of resonances from this residue with those originating from aromatic amino acid groups far from the flavin binding site. Thus, these data can again be interpreted as indicative of flavin-aromatic side chain interactions.
DISCUSSION

Flavoenzyme Classification

Massey, et al. (1969a) have proposed the classification of flavoenzymes as dehydrogenases and oxidases on the basis of oxygen reactivity, sulfite addition, and semiquinone ionization form. In the present work, we have shown that circular dichroism spectroscopy provides another basis for such a classification. Specifically, CD spectra represent a direct structural probe by means of which correlations between flavin environment and reactivity may be made. Thus, the results presented here, in conjunction with other studies (Nishikimi and Yagi, 1969; Kotaki et al., 1968), clearly establish the existence of similar flavin environments among the dehydrogenases and among the oxidases, for both the oxidized and semiquinone forms. Furthermore, even within the dehydrogenases, correlations may be made between CD spectra and redox properties (e.g., C. pasteurianum and P. elsdenii flavodoxins vs. R. rubrum flavodoxin vs. Shethna flavoprotein). These correlations seem to be based upon differences in environment which affect the ionization behavior of the flavin in its various forms and thus control its redox reactivity. (See below for further comments.) Presumably, future studies will provide further tests of this concept.
**Dithionite Reduction of the Shethna Flavoprotein Semiquinone**

We have postulated that the pH dependence of semiquinone reduction by dithionite (Fig. 47) is due to ionization of either the flavin phosphate group or the N(1) position of the flavin hydroquinone. Inasmuch as modification of the isoalloxazine ring eliminates the resistance to reduction at pH = 7.0, while removal of the side-chain hydroxyl groups does not (Table XIII), the most likely explanation seems to be that the N(1) ionization is involved and that only the anionic flavin hydroquinone can exist in the binding site. The shift of the pK of this species, from 6.2 in free FH₂ to 7.0 in the Shethna protein, could be due either to a direct protein-flavin interaction at N(1) or to inductive effects from hydrogen-bonding of the 2- and 4- position carbonyl oxygens to the protein, or both. A steric constraint on the positioning of the flavin ring in the binding site is suggested by the importance of an unhindered N(3)-protein interaction, as shown by the fact that the 3-methylFMN-apoprotein complex is completely reduced at pH = 7.0. It has been demonstrated that the presence of an alkyl group at the N(3) position does not affect the pK for N(1) ionization of free flavin hydroquinone (Ehrenberg and Hemmerich, 1966). Although no direct experimental evidence is available, the hydroquinone forms of the 2-thio and isoFMN analogs, because
of electronic effects mediated through the flavin pi system, might be expected to have a different ionization constant than that of FMNH₂. Some indication of this is given by the large differences in absorption spectra between these analogs and FMN. There is also the possibility of pK shifts through altered protein-flavin interactions.

The flavin hydroquinone has been suggested to be in a bent configuration in both its neutral and anionic forms (Ehrenberg and Hemmerich, 1968). It is thus necessary to explain how the Shethna protein can impose steric limitations preventing this non-planarity for the neutral form but not for the anionic form. This can easily be accomplished by postulating an alteration of protein structure through an electrostatic interaction between the flavin hydroquinone anion and a positively charged protein group (e.g., a protonated amino group), which would change the binding site geometry sufficiently to allow the bent flavin ring to fit. This proposed structural alteration of the protein would have to be localized, inasmuch as no major changes in peptide backbone structure were observed in the comparison of the far ultraviolet CD spectrum of the flavo-protein hydroquinone with those of the semiquinone and oxidized forms. The negatively-charged flavin hydroquinone, formed in a predominantly non-polar milieu, would also increase its solvation which could in turn cause a localized swelling of the dimensions of the binding site, thus
providing more room for the bent configuration. Proton magnetic resonance studies might be useful in providing support for these ideas.

**Oxidation of the Shethna Flavoprotein Semiquinone by Oxygen**

The neutral semiquinone form of the native Shethna flavoprotein reacts slowly with molecular oxygen \((k = 3 \times 10^{-4} \text{min}^{-1})\) at \(pH = 7.0\) to form oxidized flavoprotein and presumably the superoxide ion \((O_2^\cdot)\) as the reaction products. The rate of \(O_2\) oxidation of the unbound flavin neutral semiquinone is not known, but it is at least \(10^5\) times slower than its rate of disproportionation (Vaish and Tollin, 1970). On the other hand, the flavin anion radical reacts very rapidly with oxygen (Vaish and Tollin, 1970) with a rate close to diffusion controlled \((k = 5 \times 10^8 \text{M}^{-1}\text{sec}^{-1})\). One can account for this large difference in reactivity by assuming that oxygen reacts by removing an electron (the \(pK\) of the \(O_2^\cdot\) ion is 4.3) from the \(N(5)\) position of the flavin ring. Thus, it should be relatively easy to accomplish this with the electron-rich ring system of the anion radical, compared to the more difficult task of removing an electron from the positively charged \(N(5)\) position of the neutral semiquinone.

The difference in oxygen reactivity among the flavoenzyme neutral semiquinones could be due to the degree of protein stabilization of the protonated \(N(5)\) nitrogen.
and the negatively charged N(1) nitrogen. This stabilization seems to be reflected in the correlation we have observed between the increase in ultraviolet extinction of the flavoenzyme neutral semiquinones and oxygen reactivity (Fig. 52). The amount of stabilization is also quite sensitive to modifications of flavin structure, as evidenced by the fact that the FMN analog-Shethna apoprotein complexes are oxidized more rapidly and have a lower ultraviolet extinction than the FMN-protein complex. Modification of the flavin aromatic ring system (as with 2-thio and isoFMN) would be expected both to have a direct influence on the basicity of the N(5) position of flavin semiquinone and to affect the protein-induced stabilization. This is supported by the observation of a more rapid oxidation of the semiquinone forms of 2-thioFMN and isoFMN compared to 3-methylFMN and deoxyFMN (Table XIV). The increase in the rate of semiquinone oxidation as the pH is increased can be explained simply in terms of the postulated relation to the state of protonation of N(5) (Fig. 59), if one supposes that the oxidation of the neutral semiquinone proceeds via the anion radical at high pH.

Other types of supporting evidence for the key role of the N(5) position in determining protein semiquinone reactivity towards oxygen are ESR experiments on unbound

\[^1\text{It is not possible at present to be certain that these reactivity differences do not reflect differences in the amount of anion radical in equilibrium with the neutral radical.}\]
flavin radicals (Guzzo and Tollin, 1965) which show a high unpaired spin density at this position and the sulfite addition studies of Massey et al. (1969a). Müller and Massey (1969) have determined, from model systems, that sulfite adds to the N(5) position of the isoalloxazine ring. Only those flavoenzymes which form anion radicals can add sulfite (i.e., the oxidases). Those flavoproteins (the dehydrogenases) which form neutral semiquinones do not form sulfite adducts. These results suggest that the N(5) position is unreactive in the oxidized form as well as in the semiquinone form in the dehydrogenases. The proposal that the nature of a charged protein group in the vicinity of the flavin determines the type of semiquinone species and reactivity with sulfite (Massey et al., 1969a) cannot be proven or disproven by the present study.

**Flavin Binding to the Shethna Apoprotein**

A completely reversible resolution and recombination of flavin and protein has been accomplished, using protein spectral properties and flavin redox and spectral properties as criteria of reversibility. Obviously, a study of the effect of flavin modification on protein-flavin interactions is valid only if complete reversibility is achieved. This type of approach has the advantage of being less ambiguous than studies of the effect of chemical modification of the protein on protein-flavin interactions.
It is of interest to attempt to summarize the results of our studies in the form of a model which relates the structural features of the flavin molecule to its binding site on the Shethna protein. This would be of interest in its own right and can also serve as a guide to further studies. Such a model is shown in Figure 64. The flavin molecule is presumed to be almost completely buried in a cavity or cleft in the protein. This is based upon several lines of evidence: the large free energy of binding which implies multiple sites of flavin-protein interaction, the non-polar environment of the flavin chromophore, the fact that changes in any part of the flavin molecule cause modifications of binding and flavoprotein properties, the thermodynamic barrier to PFH₂ formation below pH 8, and the slow rate of reduction of the Shethna flavoprotein even with such a strong reductant as dithionite (implying inaccessibility of reactive sites on the flavin molecules).

Specific protein-flavin interactions are shown to occur at the 2-carbonyl and the N(3) positions, based upon the redox differences observed between the apoprotein complexes of 2-thioFMN and 3-methylFMN and the native protein. The lower binding constant of 3-methylFMN, the difference in binding between riboflavin and its 3-methyl analog, and the shift in pK of the N(3) proton in the FMN-protein complex provide further evidence for this interaction.
Fig. 64. Proposed Configuration of the FMN Molecule in Relation to its Binding Site in the Shethna Flavoprotein.

The dashed lines refer to interactions with protein groups. The enclosure suggests the over-all shape of the protein binding site.
Fig. 64. Proposed configuration of the FIV1 molecule in relation to its binding site in the Shethna flavoprotein.
One side of the binding site cavity is shown to be open with the benzene ring partly buried. The methyl groups on this portion of the flavin ring are probably important in maintaining the position of the flavin ring (suggested by the lower binding constant of isoFMN [Table XI] as well as the different redox properties of this semiquinone form relative to FMN). The rearrangement of the 8-position methyl group in isoFMN would change this interaction and generate sterically unfavorable interactions at the 6-position, thus shifting the position of the ring system. The partly open nature of this region is suggested by the ability of oxidants such as ferricyanide and cytochrome c to quickly oxidize PFH\textsuperscript{*}. This could occur via the benzene ring pi system, which has a high unpaired spin density in the flavosemiquinone (Guzzo and Tollin, 1965; Ehrenberg et al., 1967).

The side-chain hydroxyl groups are shown in close proximity to the flavin ring system within the cavity. (This is suggested for both the oxidized and semiquinone forms.) Such a concept is supported by the red-shift of the CD and difference spectra of protein-bound complexes of deoxyFMN relative to FMN (Fig. 11), by the low rotational strength of the long wavelength band in the CD of those protein-bound analogs without side-chain hydroxyl groups (Figs. 10 and 40), by the increase in long-wavelength rotational strength upon binding of flavin, by the role
of hydroxyl groups in fluorescence quenching, and by the lack of binding of tetra-O-acetylriboflavin. The low rotational strength of the long wavelength bands in the CD spectrum of the semiquinone form of deoxyFMN compared with the FMN protein (Fig. 23) suggests that this is true in the semiquinone also. The low rotational strengths of the semiquinone forms of the 2-thioFMN and isoFMN-protein complexes in the long wavelength bands (Figs. 24 and 44) suggests that those modifications position the flavin ring away from the side chain, although the CD indicates strong interactions in the oxidized form. The reduced stability of the deoxyFMN protein semiquinone provides evidence that the side-chain hydroxyl groups play a role in stabilizing the radical. This could be accomplished by interactions at N(1) which stabilize the negative charge at this position in the semiquinone (both neutral and anion forms).

The crucial importance of the phosphate group is most evident in the observation that it is essential for stable semiquinone formation. That this phenomenon is not due only to the extra binding energy is indicated by the comparison of the association constants for binding to the protein of 3-methylFMN and 3-methylriboflavin with FMN and riboflavin (Table XI). Thus, the phosphate group must act to help maintain the binding site geometry. The relative flavin fluorescence yields of the protein complexes of deoxyriboflavin and deoxyFMN compared with FMN
and riboflavin provides further evidence for different interactions in the protein complexes of the phosphorylated flavins than those which occur with the riboflavin analogs. These differences are probably to be associated with the protein moiety, inasmuch as the CD spectra of the protein-bound riboflavin analogs are quite similar to those of the FMN analogs. Still further support for this concept is to be found in the fact that bound riboflavin derivatives will form PFH₂ very readily upon reduction and will air-oxidize to PF even in the presence of an excess of apoprotein. It is interesting that a carboxyl group will not substitute for the phosphate; thus the interaction is highly specific.

The slower binding rate of the FMN analogs relative to the riboflavin analogs (Table XII) indicates that the phosphate-protein interaction is within the protein structure and not on the surface. This difference in rate has also been observed by Hinkson (1968). The rate of FMN binding increases upon lowering the pH (with an apparent pK = 7.0) (Hinkson, 1968). The ionizing group here is probably the phosphate, which, when rendered uncharged by protonation, leads to an increase in binding rate. This is in accord with the difference in rate observed between riboflavin and FMN. The ring-modified FMN analogs show essentially no differences in binding rate, while removal of the hydroxyl
groups increases the rate of binding. This suggests that the side chain is bound first (presumably through a phosphate-protein electrostatic interaction) and is then followed by the ring binding process(es).

The phosphate group also may be specifically involved in radical stabilization towards oxygen. This is suggested by the fact that fully-reduced riboflavin analog proteins do not generate radical upon air oxidation, whereas the fully-reduced FMN protein does, even at pH's greater than 8 where presumably the cavity is loosened. This could be accomplished by having the negative charges of the phosphate group in proximity to the positive charge at N(5), as shown in the model.

Finally, the model depicts the top of the cleft (shown with a portion of the ribityl side chain emerging) as relatively open to the solvent. This is necessary to explain FAD binding, since it will allow the adenosine moiety to be oriented away from the protein, as is indicated by the CD of the FAD protein and the similarity between the binding constants of FAD and riboflavin. Furthermore, it explains the pH dependence of the di-thionite reduction (if N(1) of FH₂ is involved) and the fact that the protein can be reduced at all.

Little difference in protein conformation is observed for the oxidized, semiquinone and fully-reduced forms of the Shetna flavoprotein. The principle evidence
for this is the comparison of the far ultraviolet CD spectra, the PMR spectra, and the tyrosyl ionization properties. Further support is provided by the fact that it is possible to photoreduce oxidized flavoprotein crystals to the neutral semiquinone form without disruption of the crystals. Although the protein crystals were irregular and not characterized by x-ray methods, the change of redox form within the same crystals indicates no major protein conformational changes. The ESR spectrum of a semiquinone protein crystal suspension was also identical to that obtained with the solution. These results are of interest in connection with the suggestion of a protein conformational change upon semiquinone formation based on crystallographic studies of *C. pasteurianum* flavodoxin (Ludwig et al., 1969). Although the crystals of the oxidized and semiquinone forms of this flavoprotein are isomorphous as far as cell dimensions are concerned, the diffracted intensities are changed. The authors interpret this to be due to changes in the positions of the atoms in the unit cell upon flavin reduction. However, for this protein, the semiquinone is red in the crystalline form and blue in solution, thereby directly indicating some changes in flavin environment upon crystallization.

Protein structural changes (although small) are shown by changes in the far ultraviolet CD spectra when the flavin is removed (Fig. 36). These spectral changes are too
large to be accounted for by an optically-active flavin transition in this region, even assuming a ten-fold increase in flavin rotational strength upon protein binding.

The change in tyrosyl ionization behavior upon flavin binding also indicates a structural change (Fig. 62). The possibility of tyrosyl residues at or near the flavin binding site is suggested by these results. A recent study (Hinkson, 1968) has indicated that the bound flavin protects two of the five tyrosines in the Shethna flavoprotein from nitration. Nitration of four of the five tyrosines in the apoprotein practically eliminates all flavin-binding capacity.

CD studies on the apoprotein and on the 2-thioFNM complex in the oxidized and semiquinone forms provide no clear evidence for a tryptophan-flavin interaction (Fig. 55), although the presence of a tyrosyl perturbation is suggested. Tryptophan fluorescence is quenched upon flavin binding, but the lack of any wavelength shift in the fluorescence emission spectra of the apoprotein and oxidized and semiquinone flavoproteins suggests an indirect effect (D'Anna and Tollin, 1970), perhaps mediated via energy transfer from tyrosyl groups.

Evidence for an interaction of the flavin with an aromatic residue is provided by the tailing of the long-wavelength flavin band in the difference spectra and by the changes in PMR signals due to the aromatic protons upon semiquinone formation. An unequivocal assignment of the
aromatic residue(s) as either tyrosine or tryptophan is still not possible, although at the moment tyrosine would seem to be favored.
REFERENCES


Kuhn, R. and Boulanger, P. (1936), Ber., 69, 1557.

Kuhn, R. and Wagner-Jauregg, Th. (1934), Ber., 67, 361.


Munemori, M. (1958), Talanta 1, 110.


Tollin, G. (1963a), Biochem., Z, 1720.


