Meetings

Hemes and Hemoproteins

On 16 and 17 April crystallographers, theoreticians, chemists, and biochemists convened in the library of the Johnson Research Foundation of the University of Pennsylvania for a colloquium on the Chemistry of Hemes and Hemoproteins. Many problems currently under investigation were examined in detail. The colloquium was organized to honor M. R. Lemberg, H. Theorell, D. Drabkin, and D. Goddard and especially celebrates two landmarks in the study of hemoproteins—the publication of the late David Keilin's book, *The History of Cell Respiration and Cytochrome* (Cambridge University Press) and the 40th anniversary of the work of R. Hilt and H. F. Holden on the resolution of hemoglobin [*Biochem. J.* **20**, 1326 (1926)].

In his introductory remarks M. R. Lemberg called attention to work of David Keilin and Hugo Theorell as pioneers in this field. The meeting began with a consideration of the chemical properties and physical structure of hemes. L. Hoard presented recent crystallographic evidence on the proximity of the coordinate iron of heme to the porphyrin ring structure. In particular, limits on the approach of ligands to the iron were discussed, serving as a point of controversy for subsequent discussions on the binding of water or oxygen to the heme of myoglobin and hemoglobin. In addition the limits of flexibility of the porphyrin ring were described, introducing the possibility that the commonly described planar structure need not be exclusively considered as rigid. W. Caughey, M. R. Lemberg, and R. Seyffert described recent chemical studies on the structure of heme *a* or cytoheme *a* from cytochrome oxidase. There was uniform agreement concerning the nature of seven of the eight side chains of the tetrapyrrole ring structure. These include a vinyl group, a formyl group, two propionic acid groups, and three methyl groups. The nature of the large alkyl side chain associated in position 2 of the porphyrin ring raised many questions concerning the size (C_{16} or C_{17}), degree of unsaturation, and function of the associated hydroxyl group. Caughey cited two properties of the side chain of "native" heme *a* which distinguish it from the material (cytoheme *a*) studied by Lynen and his coworkers, that is, unsaturation in the side chain and a hexoseamine-like grouping bound to the hydroxyl group of the side chain. The latter might serve as a ligand for heme *a*.

Attention was next directed toward reactions of heme with protein. H. Watson described the hydrophobic environment of the heme in myoglobin with particular emphasis on possible types of forces (London forces) that might maintain the heme in place. Of great interest was the idea that the heme unit may be puckered in some hemoproteins. Using the analogy of an open umbrella, Watson envisaged the distorted heme held by the proximal histidine (handle) with the top pointing at the distal histidine. Kinetic studies of heme interaction with apohemoglobin were described by Q. Gibson. Evidence suggesting a structural modification of the protein during the binding of the heme pointed to our lack of knowledge of the structure of globin in solution. While the arguments were presented that heme seeks a hydrophobic space, the nature of such a hydrophobic space, and perhaps its very existence in globin in solution prior to reaction with the heme, is by no means clear. The extension of these types of studies to considerations of the binding of heme *a* in cytochrome oxidase was presented by M. R. Lemberg and T. King.

Several dichotomies between the nature of ligand binding to myoglobin and to hemoglobin, as revealed by Mössbauer studies, provoked an interesting discussion. G. Lang presented evidence that the aquo compound of ferrimyoglobin could be in either the high-spin or low-spin state. Although alkaline methemoglobin was shown from Mössbauer studies to be in a low-spin state, A. Ehrenberg, on the basis of electron spin resonance data, showed that at higher temperatures the compound was a thermal equilibrium of low- and high-spin asymmetric compound.

C. Nobbs described in detail the accuracy achievable in crystallographic studies of myoglobin indicating the ability to detect a 0.1-Å movement of the iron or the rotation of the histidine about C_β-C_γ of 15 degrees in Fourier difference synthesis. Generally, the "noise" in Fourier difference syntheses of myoglobin and its derivatives amounts to approximately one-third of a carbon atom. On this basis the detection of the loss of the water molecule in the difference synthesis between ferri- and deoxyferromyoglobin is observed with accuracy sufficient to state that the "total loss" of the water molecule occurs. The nature of the water molecule attached to ferrimyoglobin crystals was discussed along lines suggested by Schonbaum (*The Enzymes*, vol. 8), Caughey, and Mildvan. This latter hypothesis suggests as an alternative to a coordinated water molecule that a coordinated hydroxide ion exists which accepts a hydrogen bond from position N_ϵ of imidazolium. (The sulfate accepts a hydrogen bond from position N_δ of the same imidazolium group.) As described by S. Maricic and M. Cohn, these structures might ultimately be tested in detail by studies of the effect of temperature upon proton relaxation times as measured by the spin-echo technique provided such studies can resolve whether movement of protons or of water molecules is involved. Nobbs also pointed to the work of Schoenborn on the location of xenon on the proximal side of the histidine and to preliminary experiments of Chance, in collaboration with Schoenborn, which indicate that the binding of xenon on the proximal side of the heme has no effect on the reactivity toward azide on the distal side of the heme.

Chance and Watson described preliminary results of a series of crystallographic studies intended to determine what structural alterations occur during the formation of the high-spin fluoride compound as compared with cyanide-ferrimyoglobin. Structurally, the fluoride compound appears to be very similar to the azide compound; no movements of any groups of the proteins are detected. Similar results have been obtained with methyl hydrogen

peroxide. These studies also reveal that the formation of the cyanide compound of myoglobin is marked by a movement of a number of groups, particularly on the distal side of the heme, but to some extent on the proximal side as well. The conformation change associated with the formation of the cyanide compound was supported by qualitatively similar but quantitatively smaller changes in the formation of the hydroxide compound. While the lack of three-dimensional data prevents drawing firm conclusions, the absence of such changes in the formation of the high-spin ferric compounds with fluoride and azide and the ferrous compounds with isocyanide suggests that criteria more subtle than spin state may be of importance in determining whether a conformation change occurs.

J. Keilin's studies on the reactions of certain nitrogenous ligands with reduced myoglobin giving a double hemochromagen spectrum, in the visible region, led to a number of questions relating to the nature of this spectrum and the relation of its components to each other and to other myoglobin derivatives.

Ligand binding to ferrimyoglobin becomes of great interest in view of the detailed structural information now available. Thus kinetic studies of soluble (Czerlinski) and crystalline (Chance) material were reported. The 20-fold decrease of reactivity of ferrimyoglobin toward azide was discussed from a number of aspects. The very early recognition by Perutz and Kendrew of the problem of ligand entry to the heme and its elaboration by Watson and Chance in terms of "breathing" of the structure of the hemoprotein crystal was discussed. This process causes a displacement of the distal histidine and other interfering groups to allow the ligand entry and affords an explanation of the reduced reactivity of the crystalline ferrimyoglobin. Thus, ligand replacement is less rapid in the crystal state than in solution because of the smaller probability of a conformation change (transitional conformation) necessary to admit the ligand. This decrease of probability can be attributed to a greater rigidity of the protein in the crystalline state, or to intermolecular contacts, particularly the GH corner of one myoglobin molecule approaching the E-chain region of the adjacent molecule. These explanations seem presently adequate to account for the observed differences of reactivity of the dissolved and crystalline material without postulating major differences of conformation of the protein structure. In a crystalline state, ferrimyoglobin hydroxide is highly unreactive toward ligand-replacement reactions, and in this case the crystallographic evidence for changes in the conformation of the hydroxide compounds provides a structural basis for this great difference of reactivity. By comparison of the reactivities toward azide of ferrimyoglobin, which contains a sulfate, and ferrihemoglobin, which does not contain a sulfate (M. Perutz, personal communication) bound to the E7 histidine, Chance believes that the sulfate attached to the histidine is not a primary cause of the lowered reactivity of ferrimyoglobin in the crystalline state. Other considerations remain, particularly the lower probability of achieving a suitable "transitional configuration" of the crystalline structure allowing the ligand to reach the active site.

F. Gurd reported on the carboxymethylation of crystalline ferrimyoglobin and on his current attempts to determine whether the distal histidine was a site of carboxymethylation. H. Watson reported that crystallographic data in three dimensions on this compound will soon be available to answer this question. The molecular interaction hypothesis of hemoglobin polymers operative in the oxygenation of hemoglobin was described by A. Schejter, after which A. Adler discussed the problems of polymer formation in the determination of the oxidation-reduction potential of heme.

The second day of the colloquium started with an examination in detail of the structure and reactivity of hydroperoxidases. T. Yonetani discussed the chemical nature of complex ES of cytochrome *c* peroxidase in terms of light and EPR absorptions as well as magnetic susceptibility. Complex ES was shown to retain two oxidizing equivalents per hematin unit, one of which appears to be retained as a free radical in the apoenzyme moiety. Peroxidase and the complex ES were obtained as large and stable crystals suitable for x-ray diffraction studies. The role of so-called "endogenous donors" in peroxidase reactions was discussed by P. Nicholls. He proposed a mechanism of peroxidase-catalyzed reactions in order to explain conflicting results reported by different investigators. In a discussion of compound III of horseradish peroxidase, whose absorption spectrum resembles that of oxymyoglobin, I. Yamazaki presented experimental evidence on the possible roles of compound III in oxidative activities of peroxidase. Although the current formulation for compound III is $Fe^{+++}O_2^-$, the hypothesis claiming the existence of molecular oxygen in the complex is yet to be proven. G. R. Schonbaum described studies on the selective modification of catalases using cyanogen bromide. His studies revealed the presence of a highly nucleophilic residue which may be situated in the vicinity of the prosthetic group, and suggest its possible participation in the reaction mediated by catalases. A. Ehrenberg described magnetic methods of investigating hydroperoxidase-catalyzed reactions, which he and H. Theorell had developed. He summarized magnetic susceptibility and EPR absorptions of hydroperoxidases and emphasized the intermediate spin states as the catalytically active forms.

Attention was then directed to studies of two purified hemoproteins functional in electron transport reactions—cytochrome *c* and cytochrome b_5. E. Margoliash described recent studies on the comparative structure of cytochrome *c* isolated from 25 different sources ranging from yeast and bacteria to heart. In particular, emphasis was placed on the invariant residues with particular reference to the polypeptide from residues 70 to 80 containing methionine. A preliminary report from R. Dickerson on the recent x-ray crystallographic study of horse heart cytochrome *c* promises that with the ability to obtain isomorphic replacement, together with the success of the preliminary x-ray studies, the detailed structure of cytochrome *c* may well be known within the next year. One approach to studying the structure of cytochrome *c* in solution was described in a detailed report of optical rotatory dispersion studies by H. Harbury. The preparation of derivatives, the comparative studies of reduced and oxidized cytochrome *c*, as well as the examination of cytochrome *c* from a variety of sources, now provide data which will help to reveal the tertiary structure of cytochrome *c*. The intriguing possibility that one of the ligands associated with the heme iron of cytochrome *c* may change in the transition from oxidized to reduced

cytochrome *c* (that is, a lysine or histidine to methionine sulfur) was suggested, but appears unlikely in view of rapid oxidation of cytochrome *c* at 34°K. The interpretation of the optical rotatory dispersion measurements of cytochrome *c* resulted in an active discussion by a number of investigators—each with his own approach to the use of these measurements for detailed examination of this hemoprotein.

P. Strittmatter then described studies on cytochrome b_5 illustrating the role of the bound metalloporphyrin in stabilizing the heme protein conformation in which a very significant portion of the amino acid residues are exposed to the medium and are chemically reactive. Heme analogs, chemical modification of the apoprotein, and the kinetics of recombination of a functional reactive cytochrome b_5 all point to the importance of heme binding in maintaining the cytochrome structure.

Ligand binding and its relationship to possible mechanisms of oxidase function was described by E. C. Slater. Discrepancies in the affinity of cytochrome oxidase for ligands such as fluoride, cyanide, or azide, as determined spectrophotometrically by alteration in absorbance with the concentrations of ligand known to inhibit the enzymatic activity, leave unresolved the mechanism of interaction. M. R. Lemberg then presented data supporting the existence of a new compound on the so-called "oxygenated" intermediate form of cytochrome a_3 of cytochrome oxidase. There is, however, no evidence at present that the molecular O_2 is contained in the compound. R. W. Estabrook discussed changes in optical absorbance spectra and correlated EPR spectra during substrate (barbiturates, steroids, and others) interaction with cytochrome P-450. The substrate-cytochrome P-450 compound formed was presumed functional during mixed function oxidation reactions. The discussion of oxidases was concluded by the intriguing results obtained by M. Kamen on CO interaction with the bacterial cytochromes RHP and O. He postulates a long-range interaction of CO and Fe that is not spectroscopically operative. The existence of two forms of CO derivatives, as revealed by kinetic analysis, points to the complexity of the reaction.

In an attempt to clarify the mechanism of electron transfer reactions in cytochrome, A. Kowalsky described an NMR study of the complexing of various paramagnetic metal ions with methionine and its derivatives in connection with the interest in methionine as a possible sixth ligand of the heme prosthetic group of cytochrome *c*. Specific interaction between the thio-ether linkage and copper, and between the thio-ether linkage and the iron of a heme octapeptide of cytochrome *c*, was detected by observation of the CH_3-S resonance. The possibility of a hyperfine contact interaction between the metal and the CH_3-S was investigated. An interesting application of the laser to the study of photosynthesis was presented by D. DeVault, who with Chance observed that a cytochrome was oxidized in *Chromatium* bacteria with a half-time of reaction of 2 μsec at room temperature after absorption of a quick pulse of laser light. On the basis of the nearly zero activation energy ($<$ 80 cal) observed at 120° to 35°K, they proposed an electron tunneling mechanism for this light-induced oxidation of the cytochrome. The existence of rapid cytochrome oxidation at 35°K limits the range of feasible reaction mechanisms.

The colloquium concluded with a discussion of theoretical aspects led by R. J. P. Williams. He succinctly defined the limits of our present understanding of the electronic structure of iron porphyrins and the need for single crystal Mössbauer and EPR data. In addition Williams described the need for more detailed information in order to extend our understanding of the role of nonligand groups of the protein and their influence on interactions with the heme. This could be obtained from ultraviolet spectroscopy and circular dichroism, but caution is necessary in interpreting the data for ligand replacement and ring or conformational changes. Subsequent to answering these questions, Williams sought answers as to how entering reactants, such as electrons, O_2, H_2O_2, and other ligands, alter the geometry of the molecules interacting. Of primary concern is the question of whether the oxidation state of the hemoprotein need only be defined in terms of iron. The contribution of M. Gouterman supported the suggestion that one must consider, from quantum mechanical calculations, not only the iron but the whole system—that is, iron, porphyrin, ligands, protein groups, and substrates.

The free exchange of data and ideas among the participants representing a variety of approaches to this study produced an intensely stimulating colloquium. It is encouraging that the current research in the area of hemes and hemoproteins retains the vigor established by the early investigators.

B. CHANCE
R. W. ESTABROOK
R. YONETANI
Johnson Research Foundation, University of Pennsylvania, Philadelphia

Leibniz Commemoration

On 12 March 1966 a symposium was held at the Polytechnic Institute of Brooklyn to commemorate the 250th anniversary of the death of Gottfried Wilhelm Leibniz, surely one of the most remarkable men who ever lived. Held in conjunction with the establishment of a new division of humanities and social sciences, it was the first of a series of symposiums on the interrelatedness of human knowledge which are designed to encourage dialogue between members of various disciplines. As planned, speakers and audience were drawn from a wide spectrum of interests, ranging at least from neurophysiology to English literature.

In the opening lecture of the morning session, chaired by D. J. deSolla Price of Yale, J. Agassi of Boston University showed that Leibniz's concepts of space and time as relations of order between things and events had been long neglected in the scientific tradition except by Kant and Boscovich. Observing that Einstein himself had held these concepts superior to Newton's concepts of absolute space and absolute time, he proceeded to show that Leibniz's ideas were partly responsible for the growth of the tradition of differential geometry and that of field theory in physics.

C. Iltis of the University of Wisconsin clarified the dispute between Leibniz and the Cartesians on the nature of the *vis viva*. She showed that it was not, as is commonly supposed, a mere battle of words in which the two concepts which we now call momentum and kinetic energy were confusingly discussed under the single concept of force, but rather a more fundamental disagreement on the nature of force itself. For Leibniz, what was real in nature was primitive force or striving, while motion