Meetings

Oxygenases

During recent years, increasing attention has been focused on the participation of molecular oxygen in biologic processes, other than energy generation in which the oxygen plays the well-known role of electron acceptor. One or two atoms of molecular oxygen may be inserted into a variety of organic substrates, these reactions being catalyzed by a group of enzymes classified as mono- and dioxygenases, respectively. Current knowledge and theory concerning the biological roles, regulation, and the catalytic mechanisms of action of these oxygenases were reviewed by a group of 35 Japanese, American, and European investigators in Kyoto, Japan, 16 to 19 May 1966, under the sponsorship of the U.S.–Japan cultural exchange program. Recent studies served as a basis for an intimate and rewarding exchange of ideas among the participating scientists.

The colloquium was opened with a discussion by K. Bloch on the mechanism of oxygen-dependent desaturation of fatty acids in illuminated or etiolated *Euglena gracilis*. This enzyme system was found not to be a true oxygenase since the oxygen, although an obligatory requirement for oleate formation, is not incorporated into any organic intermediate, but rather serves as an electron acceptor. This desaturase system has been separated into three protein components: a NADPH oxidase (nicotinamide-adenine dinucleotide phosphate, reduced form); a nonheme iron protein resembling ferredoxin; and a protein fraction, which alone has no demonstrable activity, but, together with the other components, constitutes the desaturase system. An environmental dependency of enzymic specificity becomes evident when *Euglena* is cultured in the presence or absence of light. The substrate requirement for illuminated *Euglena* is stearic acid linked by thioester linkage to the acyl-carrier protein (stearyl-ACP), whereas extracts of etiolated *Euglena* cannot utilize stearyl-ACP, but require palmityl- or stearyl-CoA (coenzyme A) as substrate.

Considerable interest has been aroused concerning the stage in protein synthesis at which the hydroxylation of proline to hydroxyproline occurs, since hydroxyproline itself is neither activated nor incorporated into protein. Important information as to the metabolic locus of proline hydroxylation was offered by A. Meister in his studies on the effect of puromycin on granuloma minces which incorporate, in vitro, labeled proline into the proline and hydroxyproline of collagen. Although puromycin markedly inhibits the synthesis of high-molecular-weight collagen, considerable amounts of hydroxyproline were found in low-molecular-weight peptides, suggesting that hydroxylation of proline occurs on the nascent polypeptide chain prior to its release from the ribosome. Adaptations in mechanisms of proline hydroxylation which have been evolved to resolve unique ecological problems are illustrated in the findings presented by N. Tamiya. Although in mammals hydroxyproline is formed only in the presence of molecular oxygen, in *Ascaris* neither anaerobiosis, CO, nor KCN inhibits hydroxyproline formation. This intestinal parasite, which lives in an essentially anaerobic environment, apparently achieves hydroxylation of proline by dehydrogenation followed by hydration, rather than by direct oxygenation.

The genetic and metabolite regulation of the enzyme concerned with *p*-hydroxybenzoate degradation in *Pseudomonas* and *Moraxella* was reviewed by R. Stanier. In *Moraxella* a substrate, protocatechuate, coordinately induces several of the enzymes that catalyze its subsequent metabolic transformations, whereas, in *Pseudomonas*, a product, β-ketoadipic acid, coordinately induces several enzymes which act to produce it from *p*-hydroxybenzoate. Induction may therefore be a direct or a feedback process. Although in the parent strain in *Moraxella* two enzymes are coordinately inducible, revertants of certain mutants manifest a noncoordinate regulation of these activities which was interpreted as being inexplicable in the framework of the traditional operon concept.

Hydroxylation of various steroids and drugs is carried out in a liver microsomal complex whose minimum components consist of a flavoprotein, a heme protein (its reduced carbon monoxide complex has an absorption maximum at 450 mμ, hence the designation P 450), and a hydroxylating enzyme. S. Tanaka reviewed data showing that this enzyme system is inducible by administration in vivo of various substrates and drugs. The rates of synthesis of the various components of this system seem to be regulated separately in that the polycyclic hydrocarbon substrates selectively enhance the activity of the hydroxylating enzyme, whereas phenobarbital induction leads to proportionate increase in both the P 450 and hydroxylating enzyme activities; in both instances there is no alteration in the amount of FAD (flavine adenine dinucleotide). That separate regulation in the biosynthesis of these various endoplasmic reticular components also occurs during development was reported by T. Yamano, who presented evidence that the neonatal elevations in P 450 and FAD were nonparallel.

A series of studies were devoted to an elucidation of the catalytic mechanisms by which hydroxylation occurs in the liver microsomal system, with particular emphasis on the function of the P 450 component. Addition of various substrates (R. W. Esterbrook) to the microsome complex alters the spectrum of the heme protein, suggesting combination of the substrate directly with or proximate to the heme. Furthermore, carbon monoxide reversibly inhibits hydroxylation by microsomes. This inhibition is reversibly counteracted by large amounts of oxygen or, in the presence of low oxygen tensions, by photodissociation, the action spectrum of which corresponds to the absorption spectrum of the P 450 heme protein. R. Sato reported that the P 450 protein exists in two equilibrium forms, each of which binds substrates irrespective of the valence state of the heme. T. Yamano described studies on the affinity of carbon monoxide for P 450 protein as a function of *p*H; these studies suggest binding of the heme iron to an imidazole group of the protein. P. Kimura de-

scribed a steroid-hydroxylating system in adrenal mitochondria which contain, in addition to the components in the liver microsome system, a ferrodoxin-like protein with a molecular weight of 22,000 and two inorganic iron and two acid-labile sulfur atoms per mole of protein. Careful electron spin resonance studies conducted by H. S. Mason excluded the presence of nonheme iron in preparations of microsomal liver hydroxylase. Thus, characteristic patterns of electron flow in the different subcellular organelles participate in hydroxylation in these tissues.

The biological role and the chemical nature of the copper-containing phenylalanine hydroxylase were reviewed by S. Kaufman. This enzyme uses molecular oxygen to convert phenylalanine to tyrosine, but, unlike other mono-oxygenases, it utilizes tetrahydrobiopterin rather than reduced pyridine nucleotide as a reductant. Purified phenylalanine hydroxylase contains no inorganic or heme iron; instead it contains catalytically essential copper atoms, which undergo oscillation in charge during the catalytic process. A. Kertesz demonstrated that polyphenoloxidase exists as a tetramer containing four copper atoms per molecular weight of 128,000; the tetramer can be converted to a catalytically active dimer by dodecyl sulfate.

Crystalline salicylate hydroxylase, lysine oxygenase, and imidazole acetate oxygenase are monooxygenases which contain no metals but do contain FAD. Although the nature of the interaction of these enzymes with oxygen is unknown, participation of the flavin moiety in the catalytic reaction is demonstrable by spectrophotometry. S. Yamamoto has shown that, under anaerobic conditions, the substrate reduces the FAD which then undergoes reoxidation by molecular oxygen. Insight into the mechanism of the conversion of salicylicate to catechol by salycilate hydroxylase was provided by M. Katagiri, who demonstrated the formation of E-FAD-S and E-FADH-S (E, protein moiety of enzyme; S, salicylate) complexes during the catalytic reaction. Thus, the activation of oxygen, before its incorporation into organic molecules, may occur without the participation of metals.

Some studies were concerned with the reaction mechanism of the dioxygenases. Metapyrocatechase, pyrocatechase, protocatechuate-4,5-oxygenase, and protocatechuate-3,4-oxygenase were all purified to the crystalline state by M. Nozaki from species of *Pseudomonas* cultured with the appropriate carbon source to induce the enzyme of interest. Metapyrocatechase has a single ferrous iron atom per mole enzyme; it seems to be bound to the enzyme with a mercaptide linkage. Pyrocatechase has two iron atoms per molecule of enzyme; addition of substrate to the enzyme under anaerobic conditions leads to modifications in absorption spectra and ESR signals suggestive of combination of the substrate with the ferric iron, which is subsequently reduced to the ferrous state. Admission of oxygen reversed these changes in spectra and ESR signals, an indication that the iron of native pyrocatechase might be in the trivalent state, but that it undergoes reduction and reoxidation during the enzyme-catalyzed oxidation of catechol.

The isolation and crystallization of 3,4-dihydroxyphenylacetate-2,3-oxygenase from induced *Pseudomonas* was described separately by both Y. Takeda and S. Senoh. The sulfhydryl reagents, *p*-chloromercuribenzoate and *o*-phenanthroline, competitively inhibit enzyme activity by displacing ferrous iron. Senoh demonstrated that the loss in catalytic activity following the addition of six equivalents of *p*-chloromercuribenzoate to the enzyme was accompanied by the release of six ferrous atoms and concomitant dissociation of the enzyme into three monomers. Thus, thio-iron linkages are concerned with both maintenance of structural integrity and catalytic function. From their ESR studies S. Senoh and T. Yamano contributed further insight into the catalytic mechanism of action of this enzyme. Addition of substrate and oxygen to the ferrous-enzyme results in the transient formation of an enzyme-Fe^{+3}–substrate-O_2 complex.

P. Feigelson reported on the catalytic mechanism and activation of purified *Pseudomonas* tryptophan oxygenase (tryptophan pyrrolase). Preparations of this dioxygenase, purified to specific activities above 10, contain both copper and tightly bound ferriprotoporphyrin. The heme participates actively in the catalysis; the heme iron undergoes oscillation in charge, being successively reduced by tryptophan and reoxidized by oxygen. Both trivalent and divalent forms of the enzyme were catalytically active, and latent enzyme preparations could be activated without net reduction of the ferriprotoporphyrin component. The heme component of the purified *Pseudomonas* tryptophan pyrrolase was converted to the pyridine

hemochrome and spectrophotometrically identified as ferriprotoporphyrin IX by Y. Ishimura. If the specific activity is 5.8 and the molecular weight is 150,000, then each mole of enzyme contains one mole of heme and 0.09 to 0.60 mole of copper. Ishimura concludes that the amounts of copper found were too small for copper to be considered a constituent of the enzyme. Resolution of the controversy concerning the presence of copper in tryptophan oxygenase awaits isolation of the enzyme in a homogeneous state.

Possible theoretical mechanisms for the oxygen-activating functions of horseradish peroxidase were considered by I. Yamazaki. He postulated that combination with peroxidase and subsequent reduction bestows upon oxygen the potential for multitudinous hydroxylation and oxidative reactions. He further purposed that, for catalysis to proceed, horseradish peroxidase and oxygenases, such as tryptophan oxygenase, must first catalyze the one-electron reduction of oxygen. These theoretical considerations may be compared with the reaction mechanism, empirically derived from tryptophan oxygenase, which involves electron transfer from tryptophan through heme to oxygen, resulting in activation of these substrates and their subsequent interaction.

The nature of the activated form of oxygen which serves as the hydroxylating agent was discussed by H. Staudinger and L. E. Orgel from experimental and theoretical viewpoints, respectively. Staudinger compared the stereochemical nature of the products of various nonenzymic model systems which generated hydroxyl radicals or oxygen atoms with those resulting from the enzymatic hydroxylation of the same substrates. In each of several instances, model systems generating oxygen atoms, but not those generating hydroxyl radicals, yielded hydroxylated products qualitatively similar to those formed by the microsomal hydroxylase system. Thus, it would seem that hydroxylations may be mediated by the generation of oxygen atoms, not by free hydroxyl radicals. Orgel reviewed the electronic nature of the high and low spin states of iron and factors influencing their ESR spectra. He also discussed theoretical and practical limitations and even the wisdom of attempting to distinguish between the various interconvertible complexes of iron and oxygen, such as Fe^{+2}--$O_2 \leftrightharpoons Fe^{+3}$--$O_2^{-1} \leftrightharpoons Fe^{+4}$--$O_2^{-2}$. He emphasized

that in metallo-oxygen complexes, the total number of electrons within the complex may be determinable but, since the charge distribution may vary continuously, it is frequently pointless to assign a discrete structure in which a particular electron distribution between the metal and the oxygen is designated.

Profound insight into the regulation and catalytic mechanisms of the oxygenases awaits future empirical and conceptual advances. It is evident, however, that this colloquium has richly contributed to these ultimate goals. The proceedings of this conference have been published in English as "Biological and Chemical Aspects of Oxygenases," edited by K. Bloch and O. Hayaishi, and may be purchased for $6.95 from Maruzen Company, 6 Tore Nichome Nihongashi, Chuoku, Tokyo, Japan.

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Forthcoming Events

February

17–18. American Psychopathological Assoc., New York, N.Y. (F. A. Freyhan, Room 1006, Cronin Research Bldg., St. Vincent's Hospital, 153 W. 11 St., New York 10011)

17–18. **Thyroid**, 3rd Midwest conf., Columbia, Mo. (Executive Director, Continuing Medical Education, M-176 Medical Center, Univ. of Missouri, Columbia)

18–22. American Acad. of **Allergy**, 23rd annual mtg., Palm Springs, Calif. (Executive Secretary, 756 N. Milwaukee St., Milwaukee, Wis. 53202)

19–23. American Inst. of **Mining, Metallurgical and Petroleum Engineers**, annual mtg., Los Angeles, Calif. (Executive Secretary, 345 E. 47 St., New York 10017)

19–25. **Biochemistry**, Chemical Inst. of Canada, conf., Ste. Marguerite, P.Q. (General Manager, 48 Rideau St., Ottawa 2, Ont.)

20–25. American Acad. of **Forensic Sciences**, mtg., Honolulu, Hawaii. (S. R. Gerber, 2153 Adelbert Rd., Cleveland, Ohio 44106)

21–24. **Offshore Exploration**, conf., Long Beach, Calif. (M. Richardson, Box 88, 2516 Via Tejon, Palos Verdes Estates, Calif. 90274)

22–24. **Biophysical** Soc., 11th annual mtg., Houston, Tex. (A. Cole, M. D. Anderson Hospital, Univ. of Texas, Houston 77025)

23–25. American **Physical** Soc., mtg., Austin, Tex. (K. K. Darrow, American Physical Soc., Columbia Univ., New York 10027)

26. **Psychoanalysis**, 5th annual conf., New York, N.Y. (D. M. Kaplan, 175 W. 12 St., New York 10011)

26–2. International **Anesthesia Research**