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STRUCTURE AND FUNCTION OF COPPER PROTEINS

Report on the fourth La Cura* Conference held at Villa Giulia, Manziana, Rome, Italy, 4—8 September 1979

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A. ORGANIZATION

The fourth of the traditional conferences on copper proteins, originally held at La Cura, but since 1974 at Manziana *, in the mountains north of Rome, was organized by Dr. B. Mondovì of Rome and Dr. J. Peisach of New York. The meeting was supported by the University of Rome, the Italian Consiglio Nazionale delle Ricerche, and the U.S. National Science Foundation.

At this latest conference the organization tended somewhat toward a more conventional format, with 70—80 participants, preplanned individual presentations, the generous use of handout material, and also the unavoidable timer. Nevertheless, the organizers and the old-time members of these conferences succeeded, with mostly outdoor sessions, in maintaining much of

* Since only the first conference was held at La Cura and subsequent ones at Manziana, where they are likely to be held in the future, it was decided to rename these meetings "Manziana Conferences".

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the relaxed informal atmosphere of the previous meetings with cypress and pine trees and the historic Villa Giulia providing the setting, and with long discussions during sessions, meals and some pleasant excursions to the history-laden surroundings.

B. GENERAL OBSERVATIONS

With three conferences on much the same topic preceding the 1979 one, it becomes possible to compare and make some more general remarks. At the present conference, with almost double the number of participants with varied background, spanning from medical education to inorganic chemistry or physics, and from as far away as Australia and the southeastern U.S.S.R., more expertise was assembled, which was clearly reflected in the breadth and depth of contributions and discussions. Despite the loss of some leisure, this circumstance made the 1979 meeting in some sense more valuable than previous ones. It also provided for additional education, and definition of issues, since now several inorganic chemists, EPR, NMR and X-ray spectroscopists were present to argue with each other and thus give the audience an impression of the decisiveness of the answers they were able to provide. It also became obvious that the same terms used may mean quite different things to colleagues in different disciplines, such as what constitutes "considerable distortion" of a tetrahedral site or a "blue" copper, as will be elaborated at the proper occasion below. While it is understandable that at a meeting on proteins containing a heavy metal, inorganic chemists and also spectroscopists of all specialties would abound, this is of course, in large part, also due to preferences of those who invite participants. One should, therefore, not go away with the impression that spectroscopy is the future of biochemistry of metal proteins, as some of the more lonely biochemists at the meeting might have concluded. Certainly, spectroscopy will always be an essential part of it, but classical preparative and analytical biochemistry and the art of producing the right sample in the right state will have to lay the foundations. Impressive examples of the clever combination of these subdisciplines were the work presented by E.I. Solomon (M.I.T.) on the active site structure of hemocyanin and that by P. Aisen (New York) on properties of transferrin. It is also most likely that it will be preparative advances which eventually will lead to answers on the ever-vexing question of metal and type-of-site stoichiometry in multi-center proteins such as ceruloplasmin or ascorbate oxidase.

The contributions, more so than at previous meetings, were of a very different nature, a blend of packages of solid information based on years of hard work; of clever and imaginative probing along new avenues; unfinished but thought-provoking experimentation often raising more questions than answering; and outright demonstration and admission of existing contradictions and dilemma. Also there was, on occasion, a flare-up of usual unsolved perennial questions, such as to the number of Cu atoms per molecule in

ceruloplasmin or ascorbate oxidase, the question of pyridoxal as a legitimate constituent of diamine oxidases, or the nature of the unusual EPR "copper signal" of cytochrome *c* oxidase; questions that also in this round of discussions found no unambiguous answers.

One question that seems to be a perennial one brought up by today's scientists who probably meet too often rather than not often enough is, "Was there anything new at the meeting?". Maybe not, at least nothing startlingly new, that journal refereeing, editing scientists, busily using the literature and the telephone, may not have seen or heard somewhere: but measured against what we knew at the time of the first meeting (1971), progress has undoubtedly been spectacular. And not to forget, that was only eight years ago! Many aspects of copper in proteins, which we were just beginning to see as problems at that time, are much more defined, if not fairly well or even well understood at this time, and we are now asking questions which we would not have been able to even formulate at that time. Similarly, new techniques which were just emerging on the horizon at the time of the first meeting, such as X-ray absorption edge and EXAFS spectroscopy and pulsed NMR and EPR (spin echo) techniques, can now claim their position among those definitely capable of making decisive contributions.

C. PRESENTATIONS AND DISCUSSIONS

(i) Blue Cu centers

With a view to progress in this last mentioned area, the 1979 meeting then also started with a session featuring these techniques. H.C. Freeman (Sydney) fittingly initiated the meeting by presenting the most recent results from his X-ray diffraction studies on the blue Cu protein, plastocyanin. The structure was originally solved at 2.7 Å resolution [1]. Further details have become available as the result of the refinement of the structure at 1.6 Å. The plastocyanin molecule is barrel-shaped. The protein backbone is organized in 8 strands, 7 of them having pronounced β-character. There is structural evidence for 35 inter- and intra-strand hydrogen bonds. This is not a large number in relation to the size of the molecule, and the stability of the molecular structure must be highly dependent on those side chains which are directed into the interior of the barrel. The Cu occupies a position at one end of the molecule, lying in a depression lined by hydrophobic residues between three strands of the protein backbone. The four Cu ligands are two histidines, one cysteine and one methionine. The Cu is not directly accessible to solvent, but is separated from the surrounding medium only by the imidazole ring of one histidine (His 87). The two Cu—His bonds are normal (2.02 Å), the Cu—Cys bond is short (2.18 Å), and the Cu—Met bond is long (2.90 Å). The estimated standard deviations of the bond-lengths are 0.05 Å. The distortion from tetrahedral geometry, in terms of both bond-lengths and bond-angles, appears "considerable". In Freeman's words, "Cu has never done this in

model compounds". On the other hand, H.B. Gray (Pasadena) felt that distortions of this magnitude were not all that unusual. The audience awaited with apprehension the very recent and still preliminary results on the structure of the reduced protein, the refinement of which at 1.7 Å is at present in progress. What are the changes in bond lengths at the Cu site? The answer is that the Cu atom moves by about 0.4 Å in a direction such that the Cu—Met bond decreases from 2.90 to 2.74 Å, while the imidazole ring of His 87 moves about 0.4 Å in a direction away from the Cu atom and towards the boundary of the molecule. These movements result in a lengthening of the Cu—His bond from 2.02 to 2.84 Å. These results relate to plastocyanin crystals at a measured pH of 5.9. At pH 4.2 the Cu—His distance increases to 3.1 Å. The pH-dependent behavior of the structure indicates a tendency of the histidine to dissociate from the metal. Freeman commented that if the apparent structural differences between the oxidized and reduced forms of plastocyanin were confirmed by the refinement calculations (in progress), then they would cast doubt on the published hypothesis that the Cu site in blue Cu proteins is a compromise between the requirements of Cu(II) and Cu(I). This was challenged by Gray and others who argued that no rearrangement of the ligands around the Cu occurred, but merely a movement of the Cu within an essentially unchanged ligand cage. The Franck-Condon reorganization barrier to electron transfer might accordingly still be minimal. Concerning the modes of e^- transfer to plastocyanin from its natural donor (cytochrome *f*) and from plastocyanin to the acceptor (P700), Freeman drew attention to two distinctive patches of highly conserved residues which had been identified on the surface of the molecule even at 2.7 Å resolution. One of these patches consists of two groups of acidic side-chains surrounding the exposed side-chain of a tyrosine, Tyr 83, which is also conserved in all known plastocyanin sequences. The second patch comprises a number of hydrophobic side chains surrounding the exposed edge of the imidazole ring of His 87. Both these patches are potential recognition or binding sites for redox partners. The shortest possible path between the Cu atom and a neighboring molecule would in fact be 6 Å via the imidazole ring of His 87. In line with the awakening consciousness about H^+ transfer accompanying e^- transfer, there was some discussion of the problem of accommodating a proton in the structure, following reduction. Interest was also expressed (in anticipation of a subsequent paper) in the relationship between the structure of plastocyanin and that of other blue Cu proteins. In Freeman's opinion, there are sufficient points of structural similarity between plastocyanin and azurin (a 25% larger protein whose structure has been solved at somewhat lower resolution by Jensen's group at Seattle) to confirm an evolutionary relationship between the two proteins which had been proposed earlier on the basis of sequence homology alone. Obviously it was of interest to have EXAFS studies on plastocyanin immediately follow Freeman's presentation.

K.O. Hodgson (Stanford) introduced the audience into some of the problems in evaluating EXAFS data. At this early stage spectroscopists' interpre-

tations are still highly dependent on the choice of model compounds. While distance information is quite unambiguous and accurate, amplitude information, from which the number of interacting ligands would have to be derived, is much less so. This situation is exacerbated by the absence of angular dependencies in EXAFS data recorded on randomly oriented samples as they have to be generally used. The Cu—Cys distance for plastocyanin (from French bean), as derived from EXAFS, is 2.11 Å. This agrees within error with the value 2.18 Å determined by X-ray diffraction on poplar plastocyanin. According to EXAFS this distance becomes 2.22 Å on reduction. It is of interest that the EXAFS results showed short Cu—S distances in all three blue proteins thus far examined, viz. azurin [2], stellacyanin and plastocyanin. However, there appears to be no evidence for a large increase in one of the Cu—His distances. In view of the discrepancy between the X-ray and EXAFS results, Freeman expressed his concern about the discriminatory power of EXAFS. He pointed out that Hodgson's model compound [3] has 1 sulfur and 3 nitrogen ligands but nevertheless gives an EXAFS spectrum very close to that of plastocyanin which has 2 ligands of each type. Hodgson emphasized that practically ubiquitous Zn impurities in proteins make information beyond 9600 eV useless, thus limiting information extractable from experimental spectra. He also predicted that analysis of metal proteins containing two or more metal centers with the metals in different environments would be extremely difficult. At this point W.E. Blumberg (B.T.L., Murray Hill) interjected a critical assessment of the evaluation of EXAFS data. He warned of undue optimism, estimating that in present-day evaluations based on models thus far studied "we fool ourselves 90% of the time", particularly when structural analogy is deduced from superposition of spectra.

H.A.O. Hill (Oxford), representing protein PMR spectroscopists, explained the pros and cons of this technique. The method usually gives only indirect information concerning the ligands and it is often difficult to distinguish ligands from other residues which are close to, but not bound to, the metal ion. The great advantage of NMR is the possibility of observing proteins in solution, so that temperature and pH dependencies can be readily monitored, as well as kinetically-determined conformers. He pointed out that conformers, as they can easily be discriminated by PMR, do not necessarily involve major changes in structure, but rather localized and subtle ones. For plastocyanins of various origins PMR spectra were closely related [4]. According to PMR both methionines present in spinach plastocyanin are close to the metal [5]. H^+ exchange in histidine residues has been a frequent object of study by PMR of proteins. In azurin [6] from *Pseudomonas aeruginosa* a histidine residue is 12 Å from the metal site, with an unusually slow H^+ exchange rate of >1 to $<35\ s^{-1}$. Hill concluded from his studies that there is a hydrophobic region close to the metal and in this pocket H^+ exchange of histidine is slowed. Hemmerich (Konstanz) suggested the possibility of a stable H^+ bond. Hill reported further that when the metal is

removed from azurin, the histidine H^+ exchange becomes more rapid, apparently by opening of the hydrophobic pocket to solvent. However, on removal of the metal, no major structural change noticeable by PMR occurs. Hill also demonstrated the usefulness of probe molecules containing substitution-inert Cr(III) complexes as relaxing agents for the blue proteins [7].

W.B. Mims (B.T.L., Murray Hill) discussed the usefulness of the nuclear modulation effect as registered by electron spin echo spectroscopy and B. Hoffman (Evanston) compared this technique to ENDOR spectroscopy, emphasizing their complementary nature. While with spin echo only weakly coupled ligand atoms can be studied, for ENDOR the converse is true and the central metal atom can also be observed. ENDOR is the more laborious procedure, but both techniques have the bonus of providing information about quadrupole couplings for the nuclei observed. With blue Cu proteins the detection of two nitrogenous ligands by ENDOR and their identification as histidine by spin echo offer particularly convincing applications [8,9]. In both of these spectroscopic approaches spectra of model compounds can be of great importance for interpretations. For example, it was pointed out by Hoffman that according to models used for the evaluation of ENDOR spectra there is the appearance of a discrepancy over the nitrogenous ligand of copper-transferrin, which is clearly indicated to be a histidine by spin echo. This once more (cf. EXAFS discussion above) brings home the lesson as to the importance of the judicious use of model compounds.

The difficulties in producing models for the blue Cu site, which mimic all and not just some of the properties of that site, another recurring theme of the Manziana meetings, were discussed by B. Bosnich (Toronto) and also briefly by P. Hemmerich (Konstanz). Bosnich unveiled, to the awe of most biochemists, an elaborate strategy as to how a model could be synthesized that was expected to show the required properties. Unfortunately the synthetic efforts were yet a few steps short of the final product, so that the audience will have to wait for the next meeting to hear the outcome. It was, however, mentioned by J. Peisach (New York) that a model compound, containing two benzimidazole ligands, one thioether sulfur-, and one mercaptide sulfur ligand in a tetrahedral Cu site (synthesized by C.K. Chang, East Lansing) fulfills the requirements.

A. Finazzi-Agrò (Rome) discussed the intrinsic tryptophan fluorescence of blue proteins. As compared to the respective apoproteins, fluorescence is quenched by the intrinsic Cu to 70% in azurin and plastocyanin and 50% in the multi-Cu proteins laccase and ascorbate oxidase. He also pointed out that the fluorescence intensity in *Rhus* laccase is correlated with the presence of the Cu absorbing at 330 nm (Type 2 according to Finazzi-Agrò), whereas I. Pecht (Rehovot) maintained that the fluorescence intensity is correlated with extent of reduction of the type 1 Cu as judged from critical and extensive anaerobic titrations of laccase [10]. He pointed out that the fluorescence changes obtained with type 2 Cu-depleted laccase can result from structural changes in the protein.

A brief but thought-provoking account of picosecond photoreduction or oxidation of a blue protein was presented by R. Bersohn (New York). An azurin solution was irradiated with pulses of 615 nm light of 0.5 ps duration and then immediately by a weaker light pulse to probe the absorption. The dissolved azurin was bleached but recovered its original absorption within 1.6 ps. Obviously the process observed is $Cu^{2+} \cdots {}^{-}S{-}R \rightleftharpoons Cu^{+} \cdots {}^{\cdot}S{-}R$. The probability of charge transfer (~0.1) during a vibration period can be calculated from the Cu—S vibration frequency and the rate of charge transfer. The extremely rapid recovery from bleaching by a light pulse implies that during reduction no major changes in geometry could have taken place. Thus the limiting event(s) in oxidation-reduction seem(s) to be conformational responses in the protein rather than a rearrangement of the ligand sphere of the metal. In this respect it would be of interest to determine whether the form of the protein present after picosecond reduction is identical to that of the protein reduced for longer periods of time. The converse experiment was also reported by Bersohn, in which a solution of reduced azurin was irradiated with laser pulses of 308 nm, a region in which the Cu^{+} of the reduced protein absorbs. The solution turned blue. It thus seems possible to generate reduced or oxidized species from blue proteins photochemically, which may find some applications in rapid kinetic studies of this or similar proteins.

Properties of a little known, small blue protein (1 Cu per molecule), mavicyanin, were reported by A. Marchesini (Torino). It is a steady companion of ascorbate oxidase. As was discussed later in the meeting, among the lores of how to prepare the best ascorbate oxidase is the recommendation to use small zucchini (eat the larger ones!) at the proper season. Then the ratio of mavicyanin to ascorbate oxidase is low, while those who think they get ahead by using large zucchini will be rewarded by finding more mavicyanin to get rid of. Mavicyanin is, in most ways, analogous to stellacyanin, even in its properties of accompanying a multi-Cu protein (laccase in the case of stellacyanin). The question was raised but not answered whether the small protein might have any function in supplying either Cu or electrons to the larger companion protein.

(ii) Non-blue Cu proteins

The session on non-blue Cu proteins, including cupreins, started with a discussion by R.M. Nalbandyan (Yerevan, U.S.S.R.) on Cu proteins in brain tissue. In addition to the Cu proteins known to be present in brain, viz., Cu—Zn superoxide dismutase (SOD) and metallothionein (see below) Nalbandyan reported on a new protein termed neurocuprein, an acidic protein (isoelectric point 3.5) of mol. wt. 10,000 with one polypeptide chain and 35% glutamic and aspartic acids. It contains 1 Cu per molecule and no cysteine. The EPR spectrum shows the hyperfine structure (hfs) typical of Cu(II) with a splitting of 14 mT. U. Weser (Tübingen) and K. Lerch (Zürich) then enlarged on the subject of metallothioneins, a field that has seen increasing

interest and activity in recent years, but which is also still plagued with controversies. This is a case in point where preparative skills may well bring the sought-for clarification. According to Weser there are two principal types of metallothioneins: The Cd-Zn protein of mol.wt. 6000 and 6 gat of metal per mole, such as, e.g., the horse metallothionein-1B, which contains its cysteines in 7 Cys—x—Cys and 3 each of Cys—Cys and Cys—x—x—Cys sequences, with a total S to metal ratio of 3 : 1; and the Cu thionein, with a 2 : 1 ratio of S : Cu. In mammals Cu thionein is predominantly found in fetal tissue. Chicken Zn-Cd thionein, e.g., can be converted to Cu thionein [11]. When this is done the cysteine to metal ratio becomes 2, indicating that this ratio depends on the nature of the metal ion. The protein structures of the thioneins show considerable homology. Weser and co-workers studied Cu thionein in baker's yeast [12,13], where subspecies of this protein occur with 1 to 10 Cu atoms per molecule and a S : Cu ratio close to 2. These species, which represent an interesting series of homologs, have the same mol.wt. and are not separated by electrophoresis. They can, however, be obtained separately by controlling their biosynthesis, since the protein is inducible by Cu. Weser and his associates' ESCA studies indicate that the sulfur is present as thiolate sulfur (RS^-) and copper as Cu(I) [13].

K. Lerch (Zürich) reported on a related protein from *Neurospora* which he discovered while studying induction of the Cu protein tyrosinase. This *Neurospora* thionein has mol.wt. 2600, 25 amino acids, 6 Cu and 7 each cysteines and serines per molecule (no disulfide) and shows obvious homology to the mammalian thioneins. The protein is specific for Cu and is able to function as Cu donor for apotyrosinase. The Cu is bound as Cu(I). As to the question of the function of this type of protein, Lerch suggested that they may serve for storage and transfer, as shown with apotyrosinase, and, maybe, at higher concentrations of metal, in detoxification. Because of its small size this protein may become an attractive object of study by a number of physical and chemical approaches, of which Lerch presented a sampling.

During the discussion of thioneins there fell the word "copper-sulfur proteins", signaling the potential analogy to Fe-S proteins. The question arose: are the Cu-S proteins e^- transfer agents like the Fe-S proteins? There is no evidence that thioneins are, and labile sulfur is not part of their structure. It seems, if one searches for Cu analogs of Fe-S proteins, that the Cys—Met—His—Cu proteins such as the blue proteins described above, are the e^- transfer agents in the Cu series rather than the Cys-Cu proteins such as the thioneins.

A masterpiece of physical explorations of an artificial Cu protein, namely Cu-transferrin, was presented by P. Aisen (New York). By clever use of superhyperfine interactions on the ^{65}Cu EPR signal produced by the natural (CO_3^{2-}) or an artificial (oxalate) anion, as required for metal binding, by the use of hybrids (there are two Cu sites in transferrin), manipulation of pH, use of D_2O as solvent and by the additional application of the modulation effect as produced with ^{12}C or ^{13}C oxalate by the electron spin echo tech-

nique, a convincing picture of the intricacies of the metal sites of this molecule was revealed [14—16]. In the history of research on transferrin the pendulum has swung forth and back several times between the hypothesis of site equivalence or inequivalence. Apparently the picture now is that the sites are equivalent only in gross terms, but there are definite differences which became manifest with more subtle probing. Aisen concluded that one site, labeled B, can occur in either one of two conformations, which accounts for many of the complications encountered. The modulation effect of ^{13}C vs. ^{12}C oxalate bound to iron transferrin provides clear evidence that the anion, which is ordinarily required for metal binding, is indeed a direct ligand of the metal.

Returning to authentic non-blue Cu proteins, M.J. Ettinger (SUNY-Buffalo) was mainly concerned with a question which was acute at the 1976 Manziana meeting: is Cu(III) involved in the reaction mechanism of fungal galactose oxidase? In part probably stimulated by the interest and discussions at the previous meeting, Ettinger and his colleagues carried out a number of what appear to be decisive experiments aimed at this point. The observations which led to the postulate of Cu(III) involvement were that, when ferricyanide is added to the enzyme, the EPR signal of Cu(II) disappears and enzymic activity increases [1]. The proposal to be tested here was, therefore, whether the enzyme would shuttle between Cu(I) and Cu(III). The enzyme, as prepared, shows 100% of its bound Cu as Cu(II) by its EPR signal. The Cu(II) is readily reducible only with the aid of mediators. After reducing the enzyme 80%, anaerobically with 0.8 equivalents of ascorbate, O_2 was added. While the proposed Cu(III) mechanism predicts that Cu(III) is produced by this reaction [17], the Cu(II) EPR signal returned 100%. Spectrophotometric-potentiometric and coulometric determinations in collaboration with G. Watts (Kettering Laboratory, Yellow-Springs) confirmed that enzymic reduction was a one-electron reaction. In addition, the measured oxidation-reduction potential, $E^{0'} = 0.300 \pm 10$ V, is inconsistent with the Cu(III) postulate. From the known O_2/H_2O_2 potential (0.290 V), one predicts that the Cu(III)/Cu(II) potential would have to be less positive than 0.290 V for the Cu cycle to occur. This potential is unlikely, given the Cu(II) ligands in galactose oxidase [18,19] and model Cu(III) peptide complexes [20]. Moreover, Cu(III) reduction reactions were simply not detected in the coulometric experiments. Low temperature EPR spectra in collaboration with R. Aasa and B. Reinhammar (Göteborg) suggested that ferricyanide diminishes the enzymatic Cu(II) signal by antiferromagnetic coupling. This presentation was followed by a brief description of X-ray absorption edge studies by W.E. Blumberg, who found no evidence for Cu(III) in such spectra, when ferricyanide was added to the resting enzyme. The proponents of Cu(III) had originally contended that Cu(III) was formed under these conditions.

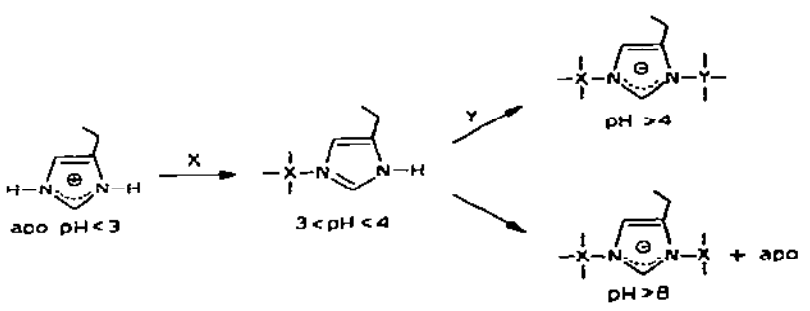
At this point it may be appropriate to insert a word of warning, namely that the rather widely used and (because of its many useful features) appreciated ferricyanide has been found in more than this case to lead to complica-

tions, since the anionic oxidizing group apparently may bind quite strongly to cationic sites on proteins.

The protein probably most popular among non-blue Cu proteins, namely SOD, was next on the program. This protein has lent itself well to a number of exercises in coordination chemistry, because it is at least not extraordinarily difficult to remove and replace, with a variety of ions, its 4 metal components, 1 Cu and 1 Zn for each of the 2 subunits. A number of tricks have been learned in the course of the years as to how Cu may be removed without removing Zn, so that, e.g., a 2 Co, 2 Zn enzyme can be produced, or how a 4 or 2 Cu enzyme can be made. L. Calabrese (Rome) told us at this meeting how to make a 2 Cu, Co, Zn, a Co, Zn and a 4 Co enzyme [21]. Obviously, if one wants to insert anything in both Zn sites, one must go via the apoprotein, since Cu is lost more readily than Zn. However, at pH 5.4 in acetate buffer, Co can be exchanged into one Zn site, even when Cu has been removed. These hybrids and variously modified SOD's, other than being curiosa, may turn out to be quite useful in obtaining information on the binding sites and also on aspects of the reaction mechanism.

J.S. Valentine (New Brunswick) presented another aspect of the intricacies of this protein's metal binding sites. It was known that the Zn-free 2 Cu protein had only half the activity at pH > 8 when compared to the native enzyme. This was interpreted as being due to changes in the coordination environment of the copper. It could be shown, however, that what actually happens on raising the pH from 5.5 to 8.5 is a disproportionation of Cu in the protein so that the empty site in a subunit becomes occupied by Cu which left its original location on another subunit, thus resulting in an equal number of completely apo- and fully occupied (by Cu) subunits [22]. At pH 5.5, however, the enzyme containing only one Cu per subunit is equally as active as the native protein, showing that the presence of the Zn in its binding site does not affect the dismutase activity. A clue to the disproportionation of the Cu was that the EPR spectrum of the 2 Cu enzyme at high pH resembled that of the 4 Cu protein [23], namely a spectrum indicating spin-spin interaction. This explains well the decrease in activity on raising the pH from 5.5 to 8.5 since Cu bound in the Zn site was previously shown not to possess dismutase activity. Valentine summarized her studies on the pH control of coordination [22,24] via a "unique binucleating ligand" by the following scheme:

Scheme I



Hill reported [25] that the backbone of SOD remains intact if the metals are removed. The rates of exchange of the C-2 protons of certain His residues, as observed with the apoprotein, are substantially reduced when the metal sites are occupied [26]. Ligand histidines can thus be differentiated from non-ligand ones. The effect depends on metal ion coordination, not on changes in protein structure. Resonances observed from His residues respond to the metal when the apoenzyme is titrated with Zn^{2+}. It can be distinguished whether one or both Zn sites are occupied. In agreement with what can be implied from Valentine's work, Hill finds that at high pH Cu no longer protects histidine from H^+ exchange. According to Hill, the fastest C-2 proton exchange of histidine ever observed, 0.07 h^{-1} at 60°C, has been seen [27] with SOD.

J.A. Fee (Ann Arbor) applied a variety of physical probes to the metal binding sites of SOD. By replacing Zn in the enzyme by ^{113}Cd, Fee and coworkers [28] were able to apply ^{113}Cd NMR, a very sensitive probe of changes in the environment of the metal. By this technique, both Zn (Cd) sites appeared to be equivalent and no significant change was seen in the Zn (Cd) site, when Cu was reduced or removed. The insensitivity of the Zn site to redox changes at the Cu site was also shown by Blumberg from studies of the X-ray absorption edge of Zn. Fee described electron spin echo results obtained in collaboration with Mims and Peisach which showed that neither high pH (12.2) (cf. ref. 29) nor CN^- breaks the Cu—His(61)—Zn bridge, while low pH does. Anions such as N_3^- and CN^- which bind to the Cu with the displacement of a water molecule [30], appear to cause a reorientation of the electronic g-tensor of the Cu such that the unpaired electron interacts strongly with only three of the four imidazole nitrogen atoms at the Cu binding site. It was also mentioned that preliminary results from single crystal EPR experiments confirmed a rotation of the g-tensor relative to the crystal axes.

A few years ago the idea had taken hold that SOD shows a "half of the sites" reactivity pattern. Apparently though, the observed kinetic behavior which largely led to this conclusion was due to preparative difficulties rather than an intrinsic property of the enzyme. A comment by M. Fielden (Surrey) on work concerning the reaction mechanism, as probed by the pulse radiolysis technique, confirmed the present stand, namely that the two active sites of the enzyme are closely similar in structure and reactivities although not fully equivalent. Fielden mentioned that the binding constants for Cu at the two Cu sites were found not to be identical.

It is doubt about what "everybody knows" and dissent that make research interesting and often lead to progress. Thus, after the long discussion on the enzyme that supposedly does away with the noxious O_2^- radical, Weser reminded the audience that the stability of O_2^- in aqueous systems is in fact very low. There are many components, including high and low molecular weight transition metal chelates as well as organic reducing agents, for example, ascorbate, which are known to react perfectly with O_2^- [31,32]. In

aprotic systems the lifetime of superoxide is substantially increased. However, in these aprotic systems such as in the lipid sections of membranes or the hydrophobic regions of many biopolymers very little or even no SOD has been found. Thus, the deleterious action of O_2^- can proceed rather uninhibited. By way of contrast to the undiminished SOD-like activity of lipophilic Cu-chelates in aqueous DMSO solutions the reactivity of native SOD is suppressed by two orders of magnitude. The question then remains, what is the physiological function of superoxide dismutases in aqueous systems?

Ironically it was left for an inorganic chemist (Hill) to come to the rescue of the enzyme (SOD) by pointing out that in considering the biological significance of an enzymatic reaction one must not look only at the reaction catalyzed and its rate; one also must keep in mind such factors as localization of the enzyme, interactions with other proteins and interfaces, i.e., aspects that have to do with the organization of the macromolecular assembly as a whole, where the protein may well have a specific role to play. Murmurs, however, were going through the audience indicating there was genuine doubt among the experts present whether nature had indeed intended this enzyme to be primarily or only a SOD, which it undoubtedly is.

A. Ehrenberg (Stockholm) discussed the Cu protein dopamine β-monooxygenase [33], which appears to be a refreshingly uncomplicated protein as compared to others heard of during the meeting: uncomplicated at least in its protein—metal relationships. The enzyme obtained from adrenal medulla, oxidizes dopamine to noradrenaline by O_2 with cooxidation of ascorbate. It has mol.wt. 290,000, has 4 peptide chains, each containing 1 Cu, which is reduced in a fraction of subunits as the enzyme is prepared. On oxidation with ferricyanide all Cu becomes EPR detectable as a single species. The redox potential of the copper is +0.360 to +0.385 V in aerobic and anaerobic titrations, respectively. Cu can be readily removed by EDTA from the oxidized enzyme (a rare phenomenon), resulting in an inactive apoenzyme. The enzyme can be reconstituted by Cu(II) and regains activity. There is no cooperativity of sites. The reduced enzyme reacts only slowly with O_2, unless a substrate, e.g., tyramine, is present. The rate of oxidation is, however, still too slow compared to the turnover rate when ascorbate is also present. The changes in the oxidation level of the copper observed by EPR, when the enzyme was going from the reduced into the steady state of the reaction, suggest that Cu is involved in catalysis.

The field of amine oxidases (plasma benzylamine and kidney diamine oxidases) was introduced by F. Buffoni (Florence). There seems to be no more controversy at this time concerning the number of Cu atoms per molecule or their non-participation in oxidation-reduction, but according to Buffoni it is still unsettled whether pyridoxalphosphate is a natural constituent of the enzyme and, if so, how many moles per mole of enzyme are present, one or two. EPR spectroscopy indicated that the two Cu sites are not entirely equivalent, though very similar. The two Cu ions are located in non-identical chemical environments, one of axial and the other of rhombic symmetry.

Present thinking is that Cu participates in O_2 reduction without undergoing any detectable valence change during catalysis. The reaction with the amine substrate is seen as a transamination of the amine with the prosthetic pyridoxalphosphate with retention of 2 electrons, resulting in release of the substrate-aldehyde [34,35]. The enzyme-bound pyridoxamine is then reoxidized by molecular O_2. This would make the need for pyridoxalphosphate plausible. B. Mondovì (Rome) briefly reported on an improved method for the preparation of bovine plasma amine oxidase, whose absorption spectrum differs from that of other preparations. The EPR spectra of the enzyme depend on pH and the observed changes indicate two different species of Cu. Mondovì expects in the near future to have a sufficient quantity of the enzyme in hand to settle, by direct analysis, the question of whether pyridoxalphosphate is present and at what ratio per mole of enzyme. This has so far been hampered by the lack of adequate quantities of enzyme sufficiently free of contamination by pyridoxalphosphate or enzymes containing it. Mondovì reported on an indirect approach to demonstrate the cofactor in the enzyme. The optical absorption spectra obtained when glycine is added to pyridoxalphosphate very much resemble those seen with the enzyme.

(iii) Binuclear copper centers

The session on binuclear Cu centers, often called type 3 Cu in the Malkin-Malmström nomenclature [36], was introduced in a masterly summary by H.S. Mason (Portland) on O_2 binding proteins in general and more specifically those containing Cu. In this survey it became apparent the enormous versatility of nature in constructing suitable sites and utilizing these in a variety of reaction types. Among prosthetic groups we find single Cu or non-heme Fe atoms, Cu or Fe pairs, heme, flavin and in some cases these groups are still unknown; as to function we find among these proteins O_2 transporters, dioxygenases, mixed-function oxidases and electron transferring oxidases. The mid-point oxidation-reduction potentials of the Cu proteins containing type 3 Cu, as far as known, lie in the range of 0.36 V for mushroom tyrosinase (2 Cu; pH 7; $n = 2$); 0.46 V for *Rhus* laccase (4 Cu; pH 7.5; $n = 2$); and 0.76 V for fungal laccase (4 Cu; pH 5.5; $n = 2$). Two of the Cu ions (type 3) are not detectable by EPR. It is generally thought that they are antiferromagnetically coupled. A characteristic of all of these proteins is an absorption band at 327—330 nm with an ϵ value of 2800—5800 M^{-1} cm^{-1}.

According to estimates by Schoot Uiterkamp et al. [37] from the EPR spectra of the NO derivatives, the Cu—Cu distance in hemocyanin (*Cancer magister*) and tyrosinase (*Agaricus bisporus*) is ca. 6 Å and a similar result was obtained by van Gelder on ceruloplasmin [38] (see however below). Mason raised the question whether these proteins contain essentially identical active sites, the proteins then possessing different domains determining the specialized function of these molecules. K. Lerch (Zürich) provided a more detailed account of *Neurospora* tyrosinase [39]. This protein shows no

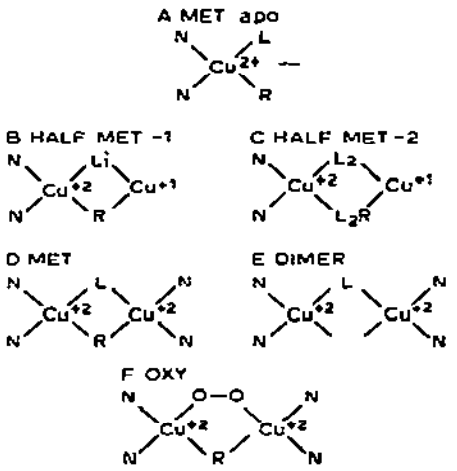
sequence homology to any other protein, as far as preliminary data indicate. There is neither a cystein-SH nor a disulfide but, curiously, there is a thioether linking Cys residue 94 to His residue 96 in position 2, which yields 2-thiolhistidine on acid hydrolysis. On dye sensitized photooxidation neither histidine nor Cu is lost, but the apoenzyme, under these conditions, loses the ability to be reconstituted with Cu, suggesting involvement of histidine in Cu-binding. The enzyme can also be reconstituted with Co (2 Co per molecule) and Cu is not able to displace Co, when Co is in the site. Co is present as Co(II). Inhibitors of tyrosinase perturb the spectra of bound Co. These observations indicate that Co is stoichiometrically bound at the active site normally occupied by Cu. His 306 is specifically destroyed by what is known as "reaction inactivation" with the substrate catechol, while one Cu is lost. With reference to the work on hemocyanin (to be discussed below): it is not known at this stage whether there is one or two bridging ligands between the two Cu atoms. According to results from the amino acid sequence the two Cu-sites cannot be completely identical.

From her long experience in the field of hemocyanins A. Ghiretti-Magaldi (Padua) gave a lucid summary of the salient properties of this pigment as found in molluscs and arthropods. Hemocyanins are known for their proneness to polymerization, which multiplies the difficulties in research, but at the same time provides for a variety of mechanisms that nature may bring into play for controlling hemocyanin function. Molluscan hemocyanin has 2 gat Cu per 50,000 dalton, whereas the arthropod pigment has 2 gat Cu per 75,000 dalton. Aggregates of various sizes are found on ultracentrifugation. Both types of hemocyanins can be crystallized. It appears that the Cu-site is more tightly organized in molluscan hemocyanin than in arthropod hemocyanin. The two Cu atoms apparently are not equally bound; one Cu is more rapidly removed by cyanide. Alluding to the theme of control of activity by quarternary interactions mentioned above, Brunori (Rome) reported that in molluscan hemocyanins with 160 sites per polymeric "molecule" functional interactions extend over domains considerably smaller than the whole polymer. Thus equilibrium and kinetic analysis indicates that in molluscan hemocyanins the "allosteric units" include approximately 12—16 sites, and within these domains cooperative effects are operational. Conformational changes occur slower than ligand binding and the rate of quarternary allosteric transitions may be correlated with the number of interacting sites per allosteric unit. Brunori pointed out the uniqueness of such systems as models to study information transfer in proteins [40].

The highlight of the session on hemocyanins was E. Solomon's (Cambridge, Mass.) information-laden 40-min "summary" of his studies on hemocyanins [41—45]. While it is quite generally impossible in the present report on the meeting to do justice to any contribution, this certainly applies to Solomon's work of which only some essentials can be given here. By a clever combination of comparative biochemistry, ligand replacements with ensuing dissociation of metal components, selective removal of one of the metals,

setting of oxidation states, light absorption, CD, IR and EPR spectroscopy and ligand field theoretical considerations, Solomon extracted a wealth of information about the metal sites of the hemocyanins. Thus a series of protein derivatives was prepared which allows the site of the copper pair to be systematically varied (Scheme II). The picture emerging of the site in oxy-

Scheme II



hemocyanin is characterized as containing two tetragonal Cu(II) ions (five coordinate with axial waters) bridged by both an endogenous protein ligand (probably phenolate) and the exogenous ligand (i.e., peroxide) with the lack of an EPR signal being the result of antiferromagnetic exchange via the endogenous bridge. The nitrogen ligands are probably furnished by histidines (histidines in hemocyanin undergo photodestruction with concomitant inactivation, as shown previously by Ghiretti-Magaldi). The tetragonal site in oxyhemocyanin is converted to a trigonal one in the deoxy form. The metal–metal distance is < ca. 3.5 Å [43,44]. If this distance increases to > ca. 5 Å the ligands joining the metals are destabilized, so that the ligand R (cf. scheme II) can be replaced by a different ligand on one of the Cu atoms. A transition dipole vector coupling model was used to assign the unique absorption spectral properties of oxyhemocyanin: the bands at 570 (abs) and 486 (CD) nm are assigned as components of the peroxide π_u^* to copper $d_{x^2-y^2}$ charge transfer. The 345 nm band is one component of the $\pi_\sigma^* \rightarrow d_{x^2-y^2}$ charge transfer. This model also predicts an end-to-end bridging geometry for the peroxide in oxyhemocyanin. The chemistry and spectroscopy of the binuclear copper active site has been compared over a series of mollusc and arthropod hemocyanins and to that of *Neurospora* tyrosinase. In general, the active sites of these metalloproteins are found to be extremely similar; however, specific differences are observed. In the hemocyanins there are quantitative structural differences including a site distortion in the

TABLE 1

Comparison of general chemical and spectroscopic properties of mollusc and arthropod hemocyanins

Spectral properties	Mollusc $\neq$ *Limulus* $\sim$ arthropods
Site instability	Arthropods but not molluscs or *Limulus*
Site distortion (Oxy $\rightarrow$ Met)	Arthropods and *Limulus* but not molluscs
Access to axial position	Arthropods $>$ molluscs $>>$ *Limulus*

arthropods which correlates with its low catalase activity and an active site instability which may be associated with intersubunit interaction. For tyrosinase all the analogous active site derivatives have been prepared and spectroscopically studied. These studies demonstrate strong similarities to oxyhemocyanin including the level of activation of the oxygen. The studies to date have indicated that the access to the active site required for associative coordination of organic substrates might be the significant variation between these binuclear "type 3" copper proteins. Table 1 summarizes comparative aspects of hemocyanin properties. Powers (B.T.L., Murray Hill) commented on EXAFS studies on *Busycon* hemocyanin [46,47]. The Cu—Cu distance in the oxyform is 3.67 ± 0.05 Å, which is short enough to allow O_2 to bind as a bridging ligand. This distance seems to decrease by 0.2 Å to 3.44 Å on deoxygenation. The absorption edge spectra are consistent with Cu(II) and Cu(I) bound to imidazole in oxy- and deoxyhemocyanin, respectively. The average nearest neighbor distance is essentially the same, viz. 1.96 Å in oxy- and 1.95 Å in deoxyhemocyanin. According to Hodgson's EXAFS results the Cu—Cu distance in *Cancer magister* hemocyanin is 3.88 Å. His model assumes four ligands per Cu. H.A. Kuiper (Rome) remarked on the fluorescence of hemocyanins. The tryptophan fluorescence is quenched by CO; there is however, evidence for emission at ca. 550 nm of a Cu—CO complex, an observation eliciting interest and surprise among the inorganic delegation present. The results of titrations and kinetic experiments using the emission band at ca. 550 nm convincingly indicate that CO binding to Cu is responsible for the new luminescence properties described [48].

(iv) Blue copper-containing oxidases

After these discussions on proteins containing Cu-sites with interacting Cu-pairs the meeting focused on that small contingent of these proteins which has the ability to reduce O_2 to water, the "blue" copper oxidases. Mondovì restated the dilemma of the variable Cu content of ascorbate oxidase: 6 Cu per molecule at Rome and 8 Cu per molecule at Konstanz. To make matters worse, these values were obtained not only on the same type of preparation (Marchesini) but also on preparations furnished to both laboratories by Marchesini. Mondovì pointed out that the EDTA-treated enzyme

as used in Rome contains 6 Cu per molecule but the specific activity of his 6 Cu enzyme appears to be the same as that of the enzyme containing 8 Cu. Similarly, L. Morpurgo (Rome) again mentioned her observation on laccase that the 330 nm absorption, which is generally attributed to type 3 Cu, also disappears on removal of type 2 Cu. Marchesini reminded the audience that ascorbate oxidase was not merely of academic interest and that its task was not only to oxidize ascorbate, but that, e.g., alkaloids of the benzylisoquinoline type are effectively oxidized by this enzyme and so are various phenolic compounds. He raised the question whether such reactions might not be involved in biogenetic processes.

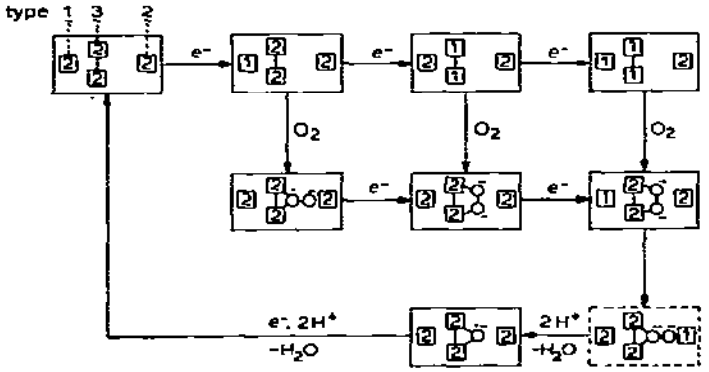
The meeting then turned to a consideration of pathways and mechanisms of electron transfer to, from and within blue oxidases, with laccase as the simplest and best understood example. Pecht recalled the previous demonstration by his group that the distribution of e^- equivalents among the redox sites of laccase depends on the redox potential of the reductant but not on its chemical nature, electrostatic charge or mechanism of transfer. Thus the Nernst plot of type 1 vs. type 3 Cu absorptions yields a slope $n = 2$ for weak or mild electron donors yet will decrease and reach the value of $n = 1$ for strong donors like $Ru(NH_3)^{2+}$ [49].

In the oxidation of reduced laccase by O_2 or H_2O_2 the above Nernst plot showed a slope of 1 [50]. The inequivalence of chemical pathways (chemical hysteresis) may be a reflection of uncoupling between the type 3 copper ion pair. A long-lived stable peroxide derivative can be prepared by reacting native oxidized *Rhus* laccase with one mole of H_2O_2 per mole. This species is characterized by an increase in the 330-nm absorption band and a typical new band in the CD spectrum at 325 nm [51]. This reaction is specific for H_2O_2 and the two oxidizing equivalents of the bound peroxide are retained by the complex. The problem addressed next was whether this laccase peroxide is also produced in the catalytic reduction of O_2. Indeed intermediates with optical (absorption and CD) properties related to those of the peroxide could be produced in several ways, viz. oxidation of half-reduced laccase with excess O_2 and under steady-state conditions with ascorbate.

Aerobic reduction of laccase with a limited amount of ascorbate leads to a transient spectrum which is possibly related to a bound O^- radical (see Scheme III). After the decay of this transient form, a stable spectrum is observed which differs from that of resting, oxidized laccase and is thought to be that of a metastable form of the oxidized enzyme. Pecht concludes from the spectra observed during turnover at low ascorbate levels that peroxy-intermediates and enzyme molecules in the metastable state account for the majority of the enzyme in the steady-state phase. He proposes the following catalytic scheme for laccase, which shows the reaction of O_2 with laccase molecules reduced to different extents and which suggests cooperation between the type 2 and type 3 sites in the O–O bond breaking reduction of the peroxide intermediate (Scheme III).

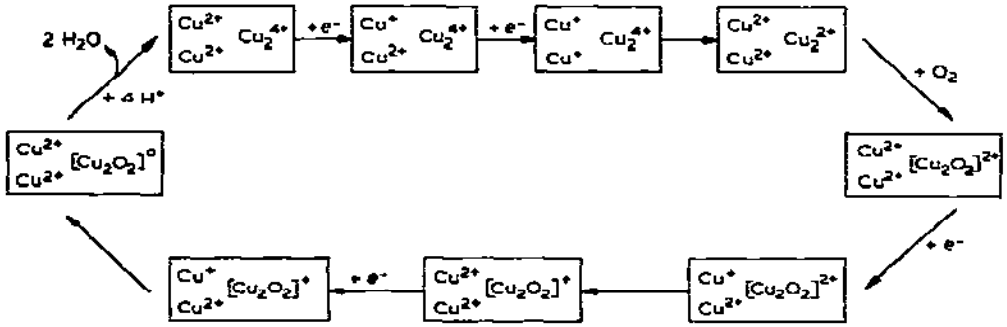
O. Farver (Copenhagen and Rehovot) communicated a determination of

Scheme III



the magnetic susceptibility of the *Rhus* laccase-peroxy complex at 300 K by the Evans NMR method. The susceptibility was found to be higher by $(1.77 \pm 0.12) \times 10^{-5}$ SI units than that of the native oxidized enzyme (3.52×10^{-5} SI units). This finding can be rationalized in terms of a decrease in the assumed antiferromagnetic coupling between the type 3 copper pair upon binding of peroxide. A coupling constant $-J = 120 \pm 10\ cm^{-1}$ was calculated, whereas $-J > 300\ cm^{-1}$ for the native oxidized protein. B.G. Malmström (Göteborg) summarized the extensive experience of his laboratory with the reaction mechanism of *Rhus* and fungal laccase in Scheme IV. In

Scheme IV



each rectangular box depicting the metal components of the enzyme the Cu in the upper left-hand corner is type 1 ("blue"), below it type 2 Cu, with the type 3 Cu to the right. (Boxes are referred to by arabic numbers, clockwise starting at upper left.) State 1 shows the fully oxidized form as isolated. In states 2 to 3 Malmström considers direct reduction of type 2 Cu by substrate possible, but continued reductions by substrate, e.g. in state 4 etc., proceed only via type 1 Cu. This delegates type 2 Cu to a role as a primer, which

becomes redundant at least for e^- transfer, once the type 3 pair has been reduced. If type 2 is blocked, e.g. by fluoride, or removed, type 1 Cu does not transfer electrons to type 3 Cu. The reaction with O_2 (states 4 and 5) is faster than re-reduction of type 1 Cu (states 5 and 6). Malmström's laboratory had previously reported the interesting finding of an intermediate in the reaction of *Rhus* and fungal laccase with O_2, which was thought to contain bound O^- [52]. This intermediate is of very short life when excess reductant is present, as it is in most experiments on the catalytic mechanism. Malmström emphasized that the events occurring in states 5—9 and back to 1 are extremely rapid and that we only know in an overall fashion what must go on, i.e. we know the overall oxidation state for the states in boxes 5—9 but the electron distribution is written in a more or less arbitrary way. Much work will be required to come to more specific and definitive conclusions as to the rapid events following the binding of O_2 to the enzyme. As will be discussed below, progress has been made in attempts to attack the analogous problem in the case of cytochrome *c* oxidase.

At the last Manziana meeting H.B. Gray (Pasadena) had reported on his strategies, following Marcus theory, to arrive at meaningful and comparable values for the electron self-exchange reaction (k_{11}) of protein redox couples including blue copper-, heme- and Fe-S protein redox sites and inorganic complexes. Gray, Mauk and Scott have now employed standard electron tunneling theory to calculate electron transfer distances from rate constants for several metalloprotein redox reactions. For proteins containing redox centers with minimal inner sphere reorganization barriers, the relationship between one-half the intersite distance (R_P, in Å) and the self-exchange rate constant at infinite ionic strength (k_{11}^{∞}) is estimated to be $R_P = 6.2 - 0.35 \ln (k_{11}^{\infty})$. Calculated R_P values based on redox reactions of heme *c*-, blue copper-, and iron-sulfur proteins with inorganic complexes support the conclusion [53—57] that hydrophobic, π-conducting ligands are able to penetrate into protein interiors, thereby reducing the distance over which electron transfer occurs. The following estimates of metalloprotein redox site-to-surface distances (ΔR_P's) have been made based on $Fe(EDTA)^{2-}$ rate data: cytochrome *c*, 3.41; cytochrome c_{551}, 4.0; plastocyanin, 2.6; azurin, 5.5; HiPIP, 5.8 Å. These kinetically-determined distances accord reasonably well with estimates of metalloprotein redox site-to-surface distances based on examination of molecular models. (The point of reference chosen was the last carbon at the edge of the heme.) The electron transfer distance in the ferricytochrome *c*—$Fe(CN)_6^{4-}$ complex has been estimated from kinetic data to be about 10 Å, which accords closely with an estimate [58] of 7—10 Å based on spectroscopic measurements.

Following up on the general theme introduced by Gray, G. Sykes (Leeds) described studies on the pH dependence of electron transfer rates, with particular attention given to the possibility of various sites on the protein being involved. The rate of reoxidation of reduced parsley plastocyanin by cobalt-tris-phenanthroline or ferricyanide was found to decrease at low pH. This

effect was, however, due to the protonation of a glutamate residue not to a change in metal-site geometry. From pH profiles obtained by PMR, Sykes concluded that *Pseudomonas* azurin was approached at the site of a certain histidine residue by cobalt-tris-phenanthroline, whereas ferricyanide interacted with a different histidine. No pH effects were seen with stellacyanin. With a protein—protein couple, viz. azurin (Cu(II)) and reduced high potential Fe-S protein (HiPIP) of *Chromatium*, no pH effects were observed. HiPIP does not use the ferricyanide interaction site when transferring an electron to azurin. With the couple plastocyanin (Cu I) and P 700 (oxidized) e^- transfer was blocked at low pH. Chance commented that one must consider the possibility of different pathways for entry and exit of electrons. Pecht recalled relevant studies done with Wherland on electron transfer between protein couples, namely plastocyanins and azurins of different origin vs. mammalian, bacterial and plant cytochromes and various cytochromes vs. each other. They concluded that "the constancy of the calculated k_{11} values is clear evidence that kinetic selectivity does not operate for any of the combinations tested" [59].

(v) Cytochrome c oxidase

Traditionally the final subject of these meetings has been cytochrome *c* oxidase, the enzyme that has attracted unexpectedly wide attention in recent years. While at the previous meetings most results presented and discussed dealt with the heme components of this enzyme, largely because they lend themselves more readily to experimentation, the 1979 meeting, with techniques and approaches developing that are applicable to Cu in cytochrome oxidase, witnessed a concentration of efforts on the Cu components. The sessions on this enzyme were duly introduced by an impressive report on the determination of the primary structure of its subunits by G. Buse (Aachen) [60—64], an undertaking that, just a few years ago, would have discouraged anybody. In beef heart oxidase Buse chemically characterized 12 polypeptides from about 8 fractions (I—VIII) seen in SDS gels. He suggests that most of the cytoplasmic polypeptides (viz., IV—VIII) are involved in anchoring the oxidase in the mitochondrial membrane. The sequences of polypeptides II, IV, VII and VIII have been completed. From the sum of the polypeptides Buse calculates a mol.wt. of 180,000 in agreement with the heme *a* content of the intact enzyme of 10—12 μmol per g of protein. Polypeptide II received particular attention, since there is good evidence that at least one copper is bound to it in the intact enzyme. There is homology between the primary structure of this subunit and that of the blue Cu proteins plastocyanin and azurin. While these two proteins show 28% homology between each other, subunit II of cytochrome oxidase has 24% homology to azurin and ca. 20% to plastocyanin. Certain regions in the sequences show striking conservation of residues, particularly that involved in Cu binding in plastocyanin, which, by analogy, can then be expected to represent a Cu

binding site in cytochrome oxidase as well. In the view of the coordination chemists present, that region Tyr—Try—Gly—Gln—Cys—Ser—Glu—Ile—Cys—Gly—Ser—Asn—His—Ser—Phe—Met— is bound to pick up a Cu^{2+} ion in solution; in view of the mitochondrial origin of this subunit Buse asked the question, why mitochondrial polypeptides of beef heart oxidase contain so much methionine (7% for subunit II) as compared to those of yeast (2%). Recent comparisons of mtDNA sequences with the sequence of polypeptide II show that mitochondria possess an altered genetic code [65,66]. The stop codon UGA codes for tryptophan and in mammalian mitochondria one codon seems to produce methionine for isoleucine. A brief discussion on genetic aspects of the sequence relationships between the small blue Cu proteins and cytochrome oxidase subunit II followed. Mason commented that the number of polypeptides present in the oxidase was not a settled matter. While he finds 7 bands by the Weber and Osborne technique, there are more than 12 seen in Swank-Munkres gels. Van Gelder remarked that by mild trypsin treatment an oxidase can be obtained that only contains subunits I, II and IV without significant change in the optical spectrum or activity. In agreement with Buse's idea that the mitochondrial polypeptides I—III constitute the enzyme proper and the findings just presented by van Gelder, Fee mentioned that there are bacterial cytochrome c oxidases, analogous to the mammalian one, which contain only two subunits. Mason then presented a sobering summary of his long-standing efforts to dissociate the subunits of the oxidase under conditions such that the bound metals do not leave their original site. He told the meeting that "every subunit of cytochrome oxidase has affinity for Cu and heme" [67]. The concentration of the dissociating detergent (SDS) as well as ionic strength are important determinants. He found mixed detergents particularly useful in preserving the native structure, e.g., a SDS—Triton X-100 mixture. Gels have to be free of Cu and must be preelectrophoresed to eliminate peroxides present which generate EPR signals. Electrophoresis was carried out at —5°C and at low current. Under such conditions band II, which appears to correspond to Buse's polypeptide II, contained all the copper and a large portion of the heme of the original enzyme. Heme *a* found in other subunits had the spectral appearance of free hematin *a*. However, in band II it had the spectrum of cytochrome *a*. Band II was green, not blue. Unfortunately to date the concentrations of material available were insufficient for EPR spectroscopy [68]. It would indeed be of the greatest interest and decisive, if heme and Cu, or a large fraction of them, were EPR silent as they are in the native enzyme, which is thought to indicate a spin coupled system with $S = 2$.

M.T. Wilson (Essex) then presented comparative biochemical data on cytochrome oxidases from some species of elasmobranchs [69]. A comparison of structural, spectral and kinetic properties between, e.g., the dogfish enzyme and the beef heart enzyme showed only minor differences. A by-product of these studies was the interesting observation that the purified oxidase from some species, e.g., hammerhead shark, occurred as a monomer,

i.e. containing four metal components, whereas it is known (cf. report of preceding meeting) that the mammalian enzyme is found as a dimeric species (containing eight metal components per dimer) under the usual conditions of experimentation and assay. This finding establishes that the dimeric species is not required for the oxidase to exhibit its known high activity. It should be noted that monomer and dimer here refer to species containing 2 Cu plus 2 heme and 4 Cu plus 4 heme groups, respectively; this must be kept in mind since arguments for the existence of an enzymatically active "monomer" containing 1 Cu and 1 heme have been brought up repeatedly. Wilson also showed data indicating that subunit II of the enzyme specifically binds Hg^{2+}. Fee elaborated further on his observations with the oxidase of *Thermus thermophilus* [70]. This enzyme is not a strict analog of the mammalian oxidase in that it contains heme *a* and copper and in addition a cytochrome *c*, presumably c_1. There are two bands after dissociation, probably one each containing one of the hemes. The stoichiometry of the components indicates that the enzyme is a c_1, aa_3, 2 Cu species, in a way, a cytochrome oxidase with built-in c_1. As expected, the enzyme readily oxidizes ascorbate with O_2. The "Cu" EPR signal shows hfs, as recently reported for the mammalian enzyme [71]. This is of great interest since it may be possible by proper isotopic substitution in the growth medium to determine the source of this hfs, which is not readily explained as arising exclusively from Cu(II).

Following these structural considerations, kinetic and mechanistic aspects were discussed. Although steady and pre-steady-state features of the reaction of cytochrome *c* oxidase (aa_3) with ferrocytochrome *c* (c^{2+}) have been extensively studied in the past, the relatively recent demonstration, by steady-state kinetic measurements, that two types of binding sites of different affinity are present on the oxidase [74,75], called for renewed study of the pre-steady-state kinetics of this reaction, so that the mechanistic implications of the presence of these sites may be recognized.

B.F. van Gelder (Amsterdam) elaborated on work concerning this problem, partly presented at previous meetings [72,73]. Pre-steady-state kinetics, as previously shown for steady-state kinetics [76], are very dependent on ionic strength, indicating that the rate of e^- transfer from c^{2+} to aa_3 is largely determined by electrostatic forces. At low ionic strength a stable 1 : 1 complex of *c* and aa_3 can be formed. In the presence of an excess of c^{2+} a single rapid second-order reaction ($k \sim 10^7\ M^{-1}\ s^{-1}$) had been observed [72], whether the high affinity site was free or occupied by *c* or by its Fe-free analog, viz. porphyrin *c*. This suggested that the low affinity site for *c* is the primary e^- accepting site on aa_3. However, the possibility existed that a more rapid initial reaction of c^{2+} at the high affinity site of aa_3 had in fact occurred, but was not resolved with the instrumentation used. In more recent work it was then observed that the pre-steady-state reaction of c^{2+} and aa_3 is indeed biphasic, with what appears to be a very rapid formation of the $c{-}aa_3$ complex ($k > 2 \times 10^8\ M^{-1}\ s^{-1}$) preceding the previously observed reaction with $k \sim 10^7\ M^{-1}\ s^{-1}$. This interpretation was supported by experi-

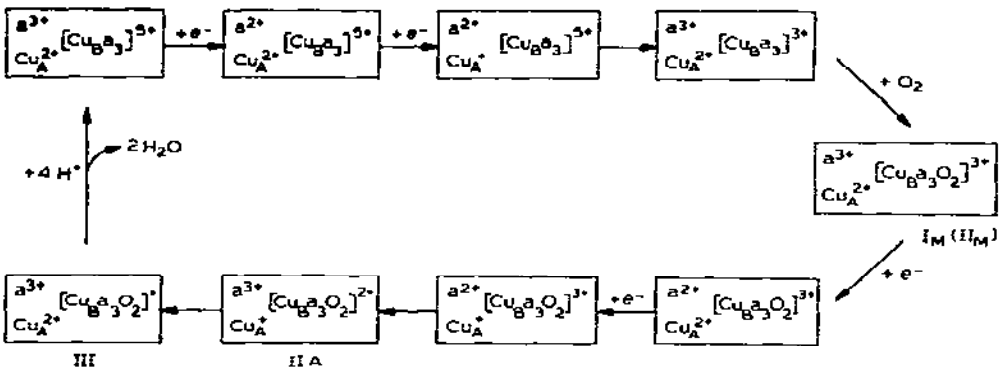
ments in which aa_3 was in excess of c^{2+}, a situation more clearly bringing out the first rapid step. Rapid e^- transfer concomitant with complex formation was indicated by the observed absorbance changes. A comparison of the rate constants, determined in the presence of an excess of c^{2+} with those found in the presence of an excess of aa_3 indicated that two slower reacting sites exist on the functional unit (2 hemes and 2 coppers) of cytochrome aa_3. On the basis of these results van Gelder discussed various models. If no site–site interactions are assumed (non-cooperative model) cytochrome aa_3 has 2 high and 2 low affinity sites available for the reaction with ferrocytochrome *c*. If, however, negative cooperativity occurs, cytochrome aa_3 has 2 high affinity sites which change into 2 low affinity sites upon binding of one cytochrome *c* molecule. On the basis of the known binding stoichiometry of *c* and aa_3 the latter model is favored or a model that incorporates features of both of these extreme cases, namely negative cooperativity between the two high affinity sites but not between the 2 low affinity sites.

Brunori reported further studies on the active form of the oxidase, the "pulsed" enzyme (cf. [77]) which now has been more extensively characterized [78]. There is a change in absorptivity at 600 nm from 10.0 mM^{-1} cm^{-1}, for the resting enzyme, to 12.2 mM^{-1} cm^{-1} for the "pulsed" enzyme. In a one-turnover situation 1 O_2 reacts per aa_3. Four oxidizing equivalents are present in the aa_3, 2 Cu units in the "pulsed" enzyme as they are in the resting oxidized form, indicating that there are no extra equivalents present from a bound oxygen species (unless Cu_B were not oxidized which is unlikely). Cytochrome *a* and Cu_A are certainly oxidized. The rate of electron transfer from *c* to *a* is identical for "pulsed" and resting enzyme. An interesting observation is that it apparently requires a measurable time span for the resting enzyme to be converted to the "pulsed" enzyme. In view of the rapid turnover of the enzyme (<1 ms) this would mean that the enzyme reaches full activation only in the course of several turnovers. Beinert commented, and Brunori agreed, that the species recently described [79] which arises within milliseconds after oxidation of reduced oxidase with O_2 and which exhibits a unique EPR signal, probably represents the same form of the enzyme. In the experiments of Beinert this species arises in <5 ms. This is, however, not in disagreement with the slow formation of this species in the course of several turnovers, since in Beinert's experiments the enzyme was reduced for several hours before being reoxidized. This would indicate that the active conformation is preconditioned in the reduced enzyme and can instantly (ms) be produced by exposure to O_2. It was noted that it has been shown [80] that the "pulsed" enzyme species (assuming identity with that of Beinert and collaborators) arises considerably later than the forms discussed in the two succeeding presentations of this session.

What was called "instantly" in the sentences immediately preceding has been analyzed in slow motion, so to speak, by B. Chance (Philadelphia) and his research group. Chance presented his latest views on the events occurring after flash photolysis of CO from the completely reduced or the mixed

valence enzyme ($a^{3+}Cu_A^{2+}a_3^{2+}$—$COCu_B^{+}$), at subzero temperatures and in the presence of dissolved O_2. As reported previously [81—84] difference spectra in the 500—650 nm region indicate intermediate states A and B which are assumed to be the O_2 (A) and peroxy (B) complexes, respectively, and C which is thought to be a mixed valence complex of the electron distribution $a^{3+}Cu_A^{2+}a_3^{2+}O_2 - Cu_B^{2+}$. Chance reported that compound C has a broad absorption in the IR (λ_{max} ca. 750 nm) and a sharp peak at 609 nm. He suggests that these features are due to Cu_B^{2+}, which leads him to the conclusion that Cu_B^{2+} has properties of a "blue" copper as discussed above. Solomon remarked that "blue" copper in its typical tetrahedral environment is not suitable for superexchange interaction as it is thought to exist between a_3^{3+} and Cu_B^{2+}. The session was concluded by a critical assessment (Malmström) of the events occurring within the cytochrome oxidase active unit (4 metals) during reduction and reoxidation by O_2. This presentation, not unexpectedly, showed many analogies to that on the turnover of the blue copper oxidase laccase as discussed above. The essence of this discussion is contained in the scheme presented below (according to Malmström) (Scheme V). As in the Scheme with laccase, electron distribution within the

Scheme V



Cu_Ba_3 pair as such or in combination with O_2 cannot be specified at present without introducing subjective choices, a difference emphasized by Malmström as compared to Chance's preferred approach. The electronic state of the noninteracting components a and Cu_A can be determined by quantitative EPR spectroscopy, as used extensively by Malmström and his group. The designation of intermediates $I_M(II_M)II_A$ and III in the scheme follows the nomenclature of Clore et al. [85].

In the subsequent, the last, session of the meeting the contributors zeroed in even more specifically on the Cu components of cytochrome oxidase. Hagen (Amsterdam) showed results of his attempts to simulate at 3 frequencies the recently described hfs pattern of cytochrome oxidase [71] with the assumption that 1 Cu ion and 1 nitrogen contribute to this hfs. Beinert

remarked that similar attempts were being made by his coauthors in collaboration with J. Pilbrow and that, according to their experience, the assumption of 1 Cu and 1 N contributing did not yield a unique solution, although 1 Cu and 1 N as a source of hfs remains a possibility. However, an unusually large splitting by nitrogen would have to be assumed. R. Wever (Amsterdam) presented a series of experiments on intramolecular electron shifts in cytochrome oxidase, extending previous results [86] that substantially added to the acceptance of the idea that 2 e^- are required to form $a_3^{2+}CO$ from the resting oxidase, i.e. acceptance of the coupled oxidation-reduction of a_3 and Cu_B [87]. Making clever use of CO and formate as ligands of a_3 and of the possibility of photodissociating CO, Wever was able to observe electron shifts which, at least in part, could be quantitatively evaluated by optical and EPR spectroscopy in terms of electrons moved and midpoint potential shifts. When the mixed valence $a^{3+}Cu_A^{2+}a_3^{2+}COCu_B^+$ complex, in the presence of formate, was exposed to light, Cu_A and a became partly reduced, particularly in the presence of formate, which is a ligand for a^{3+}. In the dark, re-reduction of a_3 and reoxidation of Cu_A and a ensued. Wever was even able to observe reduction of cytochrome c when the $a^3Cu_A^{2+}a_3^{2+}COCu_B^+$ complex in the presence of c^{3+} and formate was illuminated, thus demonstrating extensive reversal of electron flow within the cytochrome oxidase—cytochrome c system [88]. It is tempting to speculate on possible extensions of this type of approach. If a complete quantitative evaluation of such experiments became possible, precious information may be obtained on the behavior of Cu_B which has been so refractory to exploration. Since it has relevance to what will follow below, it may be mentioned that the difference absorptivity of the oxidase at 830 nm (oxidized minus reduced) was found by Wever to be 2.3 mM^{-1} cm^{-1}. Peisach then briefly mentioned an interesting new enzyme, a hydroxyindol oxidase from gill plates of molluscs, which has 8 heme groups and 8 Cu atoms in its 8 subunits that add up to mol.wt. 220,000 [89]. CO inhibits the enzyme and this inhibition is reversed by light. The enzyme has a light absorption spectrum typical of high spin heme, but shows no EPR signal. Peisach raised the question whether the heme and Cu in this enzyme are analogous to the a_3—Cu_B couple of cytochrome oxidase. The new enzyme, however, uses 1 mole of O_2 per mole of substrate and produces H_2O_2 not H_2O.

H. Beinert (Madison) then changed the pattern of presentations from that of showing results that have solved problems to one of bringing up results that create or complicate problems. His theme was a critical examination of experimental results vs. notions concerning the two Cu components of the oxidase. The question is still with us whether the so called "Cu signal" of the oxidase is indeed that of a Cu^{2+} ion. The recently demonstrated hfs pattern [71] offers no decisive solution, since it cannot be explained in a straightforward way as Cu^{2+} hfs and even if it were, this hfs might also be expressed in a signal due to a radical. Nevertheless, it still would be the signature of the Cu^{2+} ion and a quantitative evaluation of the contribution from Cu(II) could

provide valuable information on that point. Another unanswered question is: why do all workers in the field regularly find on the average 40% and not 50% of the intrinsic Cu of the enzyme in this signal? The optical properties of Cu_A will be discussed below in connection with those of Cu_B. Concerning the Cu_B, Beinert asked the audience whether they knew of any conclusive demonstration of an EPR signal from this component. Silence prevailed. Why is Cu_B not detected by EPR in states of the enzyme when both hemes are 70—90% detectable by EPR, i.e., when the $a_3^{3+}Cu_B^{2+}$ pair must largely be uncoupled? Is Cu_B still reduced in such states? He then turned to spectrophotometry as a means of detecting and determining the oxidation state of the Cu components. From a decade of experimentation with the enzyme in many states, evaluated quantitatively by EPR and low temperature optical reflectance spectroscopy, he concluded that the absorption in the near IR at 800—900 nm was within error (±10%) due to Cu_A and not to a considerable extent to Cu_B as recently proposed [90]. According to X-ray absorption edge spectra (see following presentation) Cu_A is the more covalent Cu component of the two and does not qualify as a "blue" Cu ion. Beinert, therefore, presented the dilemma that the copper with the pronounced absorption at 830 nm ($\epsilon \sim 2.3\ mM^{-1}\ cm^{-1}$) which one would usually connect with that of a "blue" copper, is not "blue", whereas the copper that has 10—15% of that absorption at 830 nm is thought to be "blue". A way out of the first part of the dilemma would be that the "Cu signal" is indeed not due to Cu(II) but to a radical in the vicinity of Cu_A and that the 830 nm absorption is then due to a (reverse) charge transfer between Cu(I) and $S^{\cdot}$, as suggested independently by Blumberg at the last and by Chan at this meeting. Referring to the increasing volume of work on cytochrome oxidase in general, Beinert cautioned that there are only one or two situations when a and Cu_A can be held in one valence state while a_3 and Cu_B are quantitatively converted into the other or vice versa. What one obtains in reality are mostly mixed situations, with oxidation states of individual components changing with time and unidentified endogenous reductants eventually coming into play (cf. [86]). Certainly, simply adding 2 e^- anaerobically to the enzyme does not lead to a clear-cut situation, as is sometimes assumed. L. Powers (B.T.L., Murray Hill) then reported on X-ray edge absorption spectroscopy to study the copper site in cytochrome oxidase [90]. Measurements were made at low temperatures (—50° to —100°C) and samples were constantly monitored by optical spectroscopy (960—520 nm) to ensure sample integrity and desired redox state of the fully oxidized (with ferricyanide), reduced +CO, mixed valence ($a^{3+}Cu_A^{2+}a_3^{2+}Cu_B^{+}CO$) and "converse" mixed valence ($a^{2+}Cu_A^{+}a_3^{3+}Cu_B^{2+}$ formate) forms. Comparison of cytochrome oxidase spectra with those of relevant copper model compounds ranging from ionic to covalent bonding and fully characterized by EPR indicate that Cu_B has a charge density similar to that of stellacyanin, a "blue" or type I copper, but does not necessarily have identical ligand groups. Cu_A has a more covalent environment than Cu_B and changes observed in reduction can be interpreted

as either a formal change in valence or a ligand change. The spectra of the mixed valence states as referred to above cannot be reproduced by addition of any proportions of the fully oxidized and fully reduced spectra indicating these are chemically distinct states. Powers emphasized that studies on model compounds show [90] that the redox state of copper cannot be determined uniquely by observation of the energy of a single transition (cf. [91]).

S.I. Chan (Pasadena) surprised the audience (at least those not familiar with ref. 92) with a set of unusual and unexpected observations on cytochrome oxidase. While it is known that the reduced enzyme forms an $a_3^{2+}NO$ complex [93], one of the few situations in which a_3 becomes EPR detectable, nobody had expected the oxidized enzyme to react with NO. Surprisingly it does, with formation of a strong, relatively axial high spin signal that can, under appropriate conditions, account for up to 70% of one heme, while cytochrome a remains 100% detectable in the usual low spin signal. The reaction with NO is fully reversed on removal of the gas. Addition of cyanide to the NO-compound deletes the high spin signal and $a_3^{3+}CN^-$ is quantitatively formed (signal at $g = 3.63$). Since Cu_A^{2+}, a^{3+} and a_3^{3+} are readily and almost quantitatively detected by EPR in signals typical of non-interacting components and optical changes in the heme absorption spectra are minor, the conclusion is that NO reacts with Cu_B to form a coupled spin system with $S = 0$. This is not changed by cyanide or fluoride, ions which react with the a_3^{3+} present. The addition of azide, however, leads to more unexpected events: the high spin heme signal disappears and a new pattern of signals emerges, including a half field signal with hfs. Chan interprets this as a triplet signal with hfs of Cu_B on the half field signal and the reaction leading to this state as: $a_3^{3+} + N_3^- + NO \rightarrow a_3^{2+} + N_2O + N_2$ with Cu_B remaining oxidized and a_3 now reduced and interacting with excess NO in its ferrous form. The triplet is thought to arise from a coupling of the two spins of 1/2 of $a_3^{2+}NO$ and Cu_B^{2+}. From these observations Chan concludes that Cu_B and a_3 cannot be separated by a histidine as had been suggested [94]. He suggests a tetragonal site for Cu_B with one of the equatorial ligands shared between a_3 and Cu_B. This ligand may be exogenous or part of the protein.

In the final presentation E. Antonini (Rome) suggested a minimal kinetic scheme with which the oxidation-reduction reactions of cytochrome oxidase can be dealt with in practice. During reduction the initial events are: $a^{3+} + c^{2+} \rightleftharpoons a^{2+} + c^{3+}$; $a^{2+} + Cu_A^{2+} \rightleftharpoons a^{3+} + Cu_A^+$ for which the rate constants are known [95]. These reactions are followed by electron transfer to the $a_3^{3+}Cu_B^{2+}$ couple, which is a relatively slow event with the isolated oxidase. a_3 and Cu_B are considered as one unit, so that e^- transfer between these components does not enter. When the a_3Cu_B couple is reduced, the reaction with O_2 is very rapid and intermediates in the reaction can be neglected. Adopting this simplified scheme and utilizing the rate constants determined for the slower steps mentioned above, M.T. Wilson, Antonini and Brunori were able to simulate progress curves of the overall reaction under various conditions satisfactorily as shown in some examples that Wilson presented.

The meeting was then concluded by Beinert in a summary of impressions, highlights, generalizations, take-home lessons, and conclusions, unfinished business and remaining dilemmas, somewhat of a short version of the present report. A sampling of opinions showed that the meeting undoubtedly was a success and that a three-year cycle for meetings on this topic leaves the right period for finishing the preliminary and recharging the stores of knowledge and the urge for communication.

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