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FROM HAEMOCUPREIN TO COPPER-ZINC SUPEROXIDE DISMUTASE: A HISTORY ON THE FIFTIETH ANNIVERSARY OF THE DISCOVERY OF HAEMOCUPREIN AND THE TWENTIETH ANNIVERSARY OF THE DISCOVERY OF SUPEROXIDE DISMUTASE

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Haemocuprein was discovered fifty years ago by T. Mann and D. Keilin as a copper protein of red blood cells, later named erythrocuprein. Superoxide dismutase was discovered twenty years ago by J.M. McCord and I. Fridovich as an enzymatic activity in preparations of carbonic anhydrase or myoglobin that inhibited the aerobic reduction of cytochrome *c* by xanthine oxidase. Astonishingly the superoxide dismutase proved to be haemocuprein. Around this time zinc was found in haemocuprein, in equimolar amount to the copper. Haemocuprein thus became copper–zinc superoxide dismutase after thirty years as an obscure cuproprotein of red blood cells. This historical article is a tribute to the achievement of J.M. McCord and I. Fridovich. Their discovery of superoxide dismutase revolutionized the study of oxygen free-radicals in biochemistry.

KEY WORDS: Haemocuprein, erythrocuprein, cupreins, superoxide dismutase, copper–zinc superoxide dismutase, superoxide, superoxide toxicity.

No discovery has played a greater part in shaping the course of oxygen free-radical research in biochemistry than the discovery of superoxide dismutase. Science advances by concepts and tools. The discovery of superoxide dismutase provided both. At a stroke it provided the concept of superoxide production and toxicity which guides oxygen free-radical research in biochemistry and, in the form of erythrocyte superoxide dismutase, a readily available enzyme, it provided a powerful tool for the investigation of superoxide-generating systems. It is fitting to commemorate great discoveries in tribute to those who made them. This year is the fiftieth anniversary of the discovery of haemocuprein by Mann and Keilin[1] and the twentieth anniversary of the discovery of superoxide dismutase activity by McCord and Fridovich.[2] Haemocuprein was a puzzling legacy of the work of David Keilin on the biochemical function of copper before it was identified as erythrocyte superoxide dismutase by McCord and Fridovich.[3] Keilin died in 1963 five years before the discovery of superoxide dismutase activity. It is ironic that superoxide dismutase turned up concealed as haemocuprein in his work on copper proteins. The enzyme was unknowable as such when Mann and Keilin discovered haemocuprein and for thirty years afterwards. Even when Keilin himself made his famous rediscovery of the myo- and histohaematin of MacMunn as cytochrome, the pigment was knowable as an intracellular respiratory catalyst. In his history of cell respiration and cytochrome, Keilin wrote: "In looking back at the development of our knowledge of respiration we can see that it did not follow a

straight or logical course".[4] Doubtless Keilin would have appreciated the remarkable course that haemocuprein was to follow in the history of biochemistry.

Haemocuprein played a unique part in the discovery of superoxide dismutase as an unknown impurity in preparations of myoglobin and carbonic anhydrase that were found to inhibit the aerobic reduction of cytochrome *c* by xanthine oxidase. When the impurity was identified as haemocuprein, a hitherto unconceived role for copper in biological systems, that was to extend to other transition metals,[5,6] was discovered. The functional versatility of copper in biological systems was known before the discovery of haemocuprein, but the protein did not fit in the paradigm of oxygen carrier, electron carrier or oxidase. It was thought to function in copper metabolism as a storage or transport protein for lack of any known enzymatic function prior to the discovery of superoxide dismutase activity.

The original isolation of haemocuprein by Mann and Keilin was a masterful exercise in protein purification and characterization in the pioneering days of protein chemistry. In 1938, Mann and Keilin observed that the copper of haemolyzed red blood cells was not dialyzable and they proceeded to the isolation and crystallization of a copper protein from ox erythrocytes that appeared to have a molecular weight of about 35,000 and to contain two copper atoms per molecule. They named the protein haemocuprein.[1] Mann and Keilin isolated a similar protein from ox liver, which they named hepatocuprein.[1] Thus was born the family of the cupreins. In 1957, Porter and Folch isolated another cuprein from bovine brain, which they named cerebrocuprein I.[7] Markowitz *et al.* renamed the cuprein from red blood cells erythrocuprein.[8] Mann and Keilin had also isolated a copper protein from horse serum (doubtless crude caeruloplasmin) which they did not distinguish from the cuprein from red blood cells, calling both haemocuprein.[1] The identity of erythrocuprein, hepatocuprein and cerebrocuprein was suspected by Porter *et al.*[9] It was established on the basis of physical, chemical and immunological evidence by Carrico and Deutsch, who proposed the general name cytocuprein for erythrocuprein, hepatocuprein and cerebrocuprien.[10] In a following paper, published in 1970, describing the presence of zinc, in equimolar amount to the copper, in cytocuprein, Carrico and Deutsch remarked: "In view of the zinc content of the protein, the name cytocuprein is not accurately descriptive. A more appropriate name should relate to the bichemical function of the protein when it becomes known".[11] We patched up the name of the protein from red blood cells by calling it erythrocyte cupro-zinc protein in work published in 1971,[12] but by this time the protein had established itself as copper–zinc superoxide dismutase.

The discovery of superoxide dismutase activity was not made by workers on the cupreins. After failing to find any known enzymatic activity in the cupreins, those in the field had been side-tracked into thinking of them as a storage or transport form of copper. This trend of thought was started by Mann and Keilin.[1] Their comments on the possible biological significance of haemocuprein and hepatocuprein are a good illustration of the way the cupreins preserved their secret for thirty years. Mann and Keilin wrote:[1]

> "The few tests to which we have submitted these compounds in order to detect some possible biological reactions or catalytic properties have so far given only negative results. We have found for instance, that the purified preparations of haemo- and hepatocuprein do not combine loosely with oxygen as does haemocyanine. They do not promote phosphorylation reactions and do not catalyse any one of the reactions catalysed by polyphenol oxidase, cytochrome oxidase,

peroxidase, catalase or carbonic anhydrase. They may still catalyse a reaction which we have not yet tested, or may require for their catalytic activity some components which have been destroyed or left behind during the process of purification of these compounds. Although these compounds do not exhibit any one of the above mentioned catalytic properties, they must nevertheless have some definite physiological significance. This is strongly supported by numerous investigations which have revealed the importance of copper in the life of different organisms, apart from its role as a constituent of haemocyanine and of polyphenol oxidase"

After stressing the biological importance of copper, Mann and Keilin went on to say:

"It is certain that some of the copper supplied to organisms with their food is utilized for building up haemo- or hepatocuprein. The formation of these compounds may therefore represent one of the steps in copper metabolism and may directly or indirectly be responsible for some of the physiological effects that are rightly ascribed to copper".

Subsequent work on the cupreins did not alter the perspective of Mann and Keilin[1] on haemocuprein and hepatocuprein. During the thirty years that the cupreins were to spend in obscurity, investigations of possible enzymatic function were concerned with oxidase activity and gave negative results.[8,13,14] Tetrazolium oxidase activity, to use the name applied to observations of inhibition of the reduction of tetrazolium salts in the presence of light, was discovered without thought of the cupreins. It was only unmasked as superoxide dismutase activity in 1973.[15] Astonishingly by 1968 copper–zinc superoxide dismutase was masquerading not only as cuprein but also as isozyme A of Brewer,[16] discovered by tetrazolium staining of starch gel electrophoretograms and misrepresented as indophenol oxidase;[17] as the tetrazolium reductase inhibitor of Fried and Fried,[18] prepared from beef liver and brain;[19,20] and as the anti-inflammatory protein of Huber *et al.*,[21] prepared from beef liver. The latter had even been found to contain both copper and zinc but was considered to be distinct from the cupreins because zinc was still unknown in these proteins.[22]

The cupreins were neglected for fifteen years after the work of Mann and Keilin.[1] In 1953, Mohamed and Greenberg described the isolation of hepatocuprein from horse liver, which they considered to be a copper storage protein as in their hands it had a variable copper content.[13] Four years later Porter and Folch added cerebrocuprein to the cuprein family.[7] The work of Markowitz *et al.*[8] and Kimmel *et al.*,[23] published in 1959, heralded the beginning of a period of activity that helped to establish the physical and chemical properties of the cupreins before they were intensively studied as copper–zinc superoxide dismutase.[8,10–12,14,23–30] Interestingly, the investigation of Markowitz *et al.*[8] was prompted by an earlier observation that erythrocytes of copper-deficient pigs had low concentrations of copper and a shortened life span in the circulation.[31] This suggested that some copper-containing component was essential to the integrity of red blood cells, as we now think of erythrocyte superoxide dismutase.[32] Erythrocuprein was duly isolated but not functional implications were drawn.[8] A subsequent study showed that total erythrocyte copper and erythrocuprein concentration, determined by quantitative immunoprecipitation, in cases of Wilson's disease and idiopathic hypocupraemia of infancy with low serum copper concentrations, were not unequivocally reduced. Erythrocuprein concentrations were also found to be remarkably constant in normal subjects.[26] Further work in which erythrocuprein concentrations were measured by radial imm-

unodiffusion showed that little variation in erythrocuprein concentration occurred in various physiological conditions and disease states.[33]

Shields *et al.* observed some labelling of erythrocuprein with ^{64}Cu in whole blood.[26] In a study of the labelling of carbonic anhydrase isozymes with ^{65}Zn in haemolyzed blood, Funakoshi and Deutsch observed some labelling of erythrocuprein with ^{65}Zn, which they interpreted as an exchange of erythrocuprein copper with ^{65}Zn.[34] Subsequent analysis of this interpretation led to the discovery of zinc as an integral component of erythrocuprein (cytocuprein) described by Carrico and Deutsch in 1970.[11] By this time the superoxide dismutase activity of erythrocuprein had been discovered.[3] Some doubt as to the physiological significance of this surprising activity was at first expressed. In their paper describing zinc in cytocuprein, Carrico and Deutsch remarked: "McCord and Fridovich [Reference 3] have reported that cytocupreins from bovine and human erythrocytes possess superoxide dismutase activity, but the physiological significance of this activity is uncertain. Possibly the zinc in cytocuprein is related to this biochemical function".[11]

When the superoxide dismutase activity of erythrocuprein was discovered,[3] we were engaged in physico-chemical studies of the protein[12,29,30] resulting from an interest in the elucidation of the metal ligands in copper proteins by means of photooxidation.[35,36] We did not immediately grasp the biochemical significance of the discovery of superoxide dismutase activity in erythrocuprein. When we reported our work on the isolation and characterization of bovine erythrocuprein, which we called erythrocyte cupro-zinc protein, we remarked:[12]

> "The biochemical function of erythrocyte cupro-zinc protein is enigmatic. The recently discovered superoxide dismutase activity [Reference 3], which has been confirmed in the present preparation of the bovine protein, is a puzzling ferature of the protein. The activity is due to the presence of copper, since it is found in apoprotein reconstituted with this metal but not in apoprotein reconstituted with zinc [Reference 3]. The metals in the protein do not exchange freely with ^{64}Cu [Reference 26] or ^{65}Zn [Reference 11]. They may represent a stable pool of copper and zinc which is only turned over with the red blood cells".

In retrospect the presupposition of a metal storage pool was unfounded, but the idea of metal storage had become so engrained in work on the cupreins before the discovery of their superoxide dismutase activity that it was difficult to relinquish it. The conjecture of Carrico and Deutsch[11] that the zinc might be related to the superoxide dismutase activity of erythrocuprein is remarkable when viewed against present knowledge of structural and functional relationships in copper–zinc superoxide dismutase. We proposed a structural role for the zinc from circular dichroism studies, suggesting that the metal stabilized the tertiary structure of the protein.[30] The discovery of a histidine residue, histidine 61 in the amino acid sequence of bovine copper–zinc superoxide dismutase, acting as a bridge between the copper and the zinc, eventually led to present views which regard the zinc as an enhancing factor in the detailed mechanism of copper–zinc superoxide dismutase.[32,37,38]

It is doubtful whether the true function of the cupreins could have been discovered in the field of the cuproproteins in the 1960s. To the extent that they were studied, the proteins then appeared to have no redox function. The influential review of Brill *et al.* on copper in biological systems, published in 1964, excluded redox reactions for the cupreins.[39] The review was remarkable for its insight into the structure of the copper site in the cupreins from simple spectroscopic considerations. In considering-model copper complexes, Brill *et al.* wrote:[39]

"A further interesting case is that of the low-symmetry, tetradentate ligand, bisphenylendiamine salicylaldehyde, which can wrap around the cupric ion leaving one side open. Such complexes may resemble cuproproteins such as erythrocuprein, for the extinction coefficients (> 100) and wavelength ($\sim 600\,m\mu$) are similar".

Their summing up of the cupreins was:

"Members of [this] group are characterized by an EPR spectrum not very different from the simple inorganic complexes of tetrammine type. Their low extinction coefficients, < 500, suggest that the absorption bands are due to *d-d* transitions, though the values are sufficiently great to demand high asymmetry of the ligands such as is provided by 'wrap-around' ligands like bissalicylaldehyde ethylene diamine. The suggestion that there are roughly four nitrogen donors binding the cupric ion could explain the apparently low redox potential. The proteins undergo no redox reactions"

We now know that the redox potential of the copper is unusually high, not low, because of the presence of the zinc and the tetrahedral distortion of the copper ligands produced by the protein conformation. The copper undergoes simple redox cycling with superoxide radicals in the formal mechanism of the enzymatic reaction. The active site is structured to enhance the already high rate of spontaneous dismutation of superoxide radicals, which it does by several orders of magnitude. The metals lie close to each other at the bottom of a deep cleft on the outside of an eight-stranded β-barrel, between two large loops of non-repetitive structure. The zinc is completely buried. The copper is slightly exposed.[37,38] The protein fabric can indeed be imagined as being wrapped around the copper leaving one side open, as envisaged by Brill *et al.* in 1964.[39]

The inhibitory effect of preparations of myoglobin[40] or carbonic anhydrase[41] on the reduction of cytochrome *c* by xanthine oxidase in the presence of oxygen, that led to the discovery of superoxide dismutase, was observed by Irwin Fridovich in the 1960s. The original interpretation of this observation assumed that xanthine oxidase was capable of the univalent reduction of molecular oxygen to superoxide, as deduced by Fridovich and Handler.[42] Cytochrome *c* was considered as a substrate of xanthine oxidase reduced by enzyme-bound superoxide in a reaction inhibited by binding of myoglobin or carbonic anhydrase. Kinetic studies appeared to support this interpretation. The reduction of cytochrome *c* showed saturation kinetics, and the inhibitory effect of myoglobin or carbonic anhydrase preparations was competitive with respect to cytochrome *c*. Superoxide dismutase activity was discovered in 1968 when Joe McCord, in the laboratory of Irwin Fridovich, undertook the investigation of the competitive action of carbonic anhydrase preparations.[2,43–45] The observed inhibition had an apparent K_i of the order of 10^{-9} M. This suggested a high affinity of xanthine oxidase for carbonic anhydrase, but no direct interaction of xanthine oxidase with carbonic anhydrase could be demonstrated in equilibrium binding studies. It was at this impasse that McCord conceived the liberation of superoxide radicals into free solution in the xanthine oxidase reaction. This was a bold hypothesis. McCord thought of univalently reduced oxygen as a carrier of electrons between xanthine oxidase and cytochrome *c* by analogy with methylene blue, which could be considered to act in free solution, after still observing saturation kinetics for the reduction of cytochrome *c* by xanthine oxidase in the presence of the dye instead of oxygen. He perceived that with liberation of superoxide in free solution, K_s for the aerobic reduction of cytochrome *c* by xanthine oxidase should depend on the steady state

concentration of superoxide and therefore on the concentration of xanthine oxidase. This was indeed observed for K_s for cytochrome *c*, and K_i for carbonic anhydrase or myoblobin, in contrast to K_s for xanthine which did not change with xanthine oxidase concentration. McCord argued that under steady state conditions the concentration of free superoxide, and therefore the initial rate of reduction of cytochrome *c* at concentrations with negligible effect on the steady state level of the radical, should vary with the square root of the concentration of xanthine oxidase. His observation that K_s for the reduction of cytochrome *c* increased with xanthine oxidase concentration provided him with a set of non-intersecting Lineweaver-Burk plots from which he obtained initial reduction rates at a cytochrome *c* concentration of 5×10^{-8} M by extrapolation. These rates were directly proportional to the square root of xanthine oxidase concentration, as he had expected. His data also permitted him to verify the prediction that at saturating concentrations of cytochrome *c* the initial rate of reduction should be directly proportional to the concentration of xanthine oxidase. The approach was indeed remarkable. As McCord himself has put it:[43]

> "By a somewhat unconventional examination of certain kinetic parameters we were led to the conclusion that milk xanthine oxidase effects the univalent reduction of molecular oxygen to form the unstable intermediate O_2^-, the superoxide free radical anion, and that this species is released into free solution by the enzyme and is the species responsible for the reduction of ferricytochrome *c* [Reference 2]".

The ability of xanthine oxidase to release superoxide into free solution was independently, and more conventionally, confirmed in contemporaneous work by Knowles *et al.*, published in 1969, in which the electron-parmagnetic-resonance signal of the superoxide radical trapped by a rapid-freezing technique, was observed at high pH.[46] Knowles *et al.* pointed out that superoxide radical produced by xanthine oxidase need not remain bound to the enzyme, but their work under alkaline stabilizing conditions was not conducive to thinking of the fate of the superoxide radical under biological conditions.

At first it was thought that carbonic anhydrase or myoglobin could facilitate the dismutation of superoxide but McCord quickly found that an inhibitor of cytochrome *c* reduction by xanthine oxidase, present in very small quantities, could be separated from carbonic anhydrase preparations by anion-exchange chromatography on DEAE-cellulose, and from myoglobin preparations by gel filtration on Sephadex G-75. The amount of inhibitor observed in relation to total protein suggested an apparent K_i of the order of 10^{-11} M and the significance of the inhibitor was immediately realised. Of this stage in the discovery of superoxide dismutase, McCord himself has written:[43]

> "Since the inhibitor was effective at 10^{-11} M, a stoichiometric consumption of O_2^- could be ruled out. This suggested the hypothesis that this protein was catalyzing the destruction of O_2^- radicals in free solution, probably by the well documented reaction given by [$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$]. Therefore we proposed the existence of a new enzyme, 'superoxide dismutase' ".

When the new enzyme was isolated by enrichment of the inhibitory activity on the reduction of cytochrome *c* by xanthine oxidase observed in haemolyzates of bovine erythrocytes, the purified protein obtained after fractionation with organic solvents and anion-exchange chromatography on DEAE-cellulose turned out to be haemocuprein.[3] The protein had in fact been contaminating carbonic anhydrase prepared quite similarly.

New enzymes are fundamental discoveries to the extent that they permit funda-

mental generalizations in biology. The discovery of superoxide dismutase focussed attention on the univalent pathway of oxygen reduction in biological systems. Superoxide production was seen as an inevitable consequence of oxygen utilization or exposure to oxygen of living organisms. The superoxide radical was perceived as a toxic oxygen species in biology. True enough free-radical ideas of oxygen toxicity appeared before the discovery of superoxide dismutase, prompted by observations of similar effects of oxygen poisoning and ionizing radiation on living organisms,[47] but these ideas did not pin down the superoxide radical as the root-cause of oxygen toxicity. Enzymatic activites are pointers to the logic of living cells. In so far as we understand this logic, biological catalysis of superoxide dismutation points to accelerated elimination of a toxic oxygen species.[5,6,48,49]

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