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Article

The Enigmatic Metallothioneins: A Case of Upward-Looking Research

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Abstract: In the mid-1950s, Bert L. Vallee and his colleague Marvin Margoshes discovered a molecule known today as metallothionein (MT). Meanwhile MTs have been shown to be common in many biological organisms. Despite their prevalence, however, it remains unclear to date what exactly MTs do and how they contribute to the biological function of an organism or organ. Honoring Dr. Vallee's sometimes innovative approach to research, this contribution sets out to show how philosophy of science can help us gain a clearer picture of biochemical research. We shall look into both the discovery of as well as recent research on Dr. Vallee's beloved family of MT proteins to illustrate (i) how exploratory and upward-looking research play important roles in biochemical discoveries although they do not fit the paradigmatic approach of decomposition and structure-function mapping. Besides, we shall suggest (ii) that while other biochemical molecules exhibit a clearly identifiable function, other research hypotheses might be worthy of pursuit in the case of MTs.

Keywords: Metallothionein (MT); Scientific discovery; Scientific pursuit; Research strategies; upward looking research; Exploratory research; Protein function

1. Introduction

After the second world war, several scientists have been interested in studying the metabolism of metals, especially iron, zinc, and copper in biological organisms. Bert L. Vallee, who worked at Harvard Medical School and MIT at the time, was a pioneer in the field. After he graduated from New York University in 1943, he recognized the potential of spectroscopy to detect metals in biological systems. Regarded as a genius by his colleagues and known for sometimes rigorous questioning of assumptions, Dr. Vallee (as he preferred to be called) had already been reporting extensively about alcohol dehydrogenase, a metalloprotein [1]. In the mid-1950s, Vallee came across translations of two publications form Doklady Akademii Nauk SSSR (the official journal of the USSR Academy of Science) reporting cadmium in several biological systems such as the Aspen tree, algae and other marine species as well as amphibians, reptiles, and even mammals [2]. At first, Dr. Vallee was skeptical about the validity of the reported data. For one thing, cadmium "never has been demonstrated to be an integral part of a natural product." (1957, p. 4813) [3]. For another, A.O. Vinar (the author of one of those manuscripts) neither mentioned any references in his paper, nor did he use established methodology. Instead, Vinar reported the results of self-developed colorimetric and spectrographic techniques to measure the amount of cadmium in biological systems. Adding to this potentially problematic methodology, Vallee failed to reproduce some of the experimental results Vinar had reported. For instance, Vallee was unable to induce hypoglycemia in a mammalian species upon injection with cadmium chloride in his own laboratory [2]. Still,

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Vallee's interest was sparked. After all, he had been studying zinc in biological organisms for years and he could not fail to notice that zinc and cadmium are chemically very similar substances: "The conjoint presence of cadmium and zinc in zinc ores, the difficulties encountered in separating them and ascertaining their identities by chemical means implied that biological matter might discriminate less between their absorption, utilization and perhaps even function than might be thought. Hence, I decided to search for cadmium proteins and enzymes, their biological roles, normal and abnormal metabolism, much as I had proceeded to study the biological role of zinc." (1978, p. 24) [4] So, Vallee set out to search for cadmium in mammals himself. Together with Marvin Margoshes, he analyzed tissues of human, horse, cow, hog, and sheep kidneys. In 1957, Vallee and Margoshes published a report stating that they actually did identify cadmium in these species [3]. They continued to examine the kidney cortex of equine, which seemed to contain relatively high amounts of cadmium, to try and identify what chemical compound cadmium is part of in the kidney cortex. While the exact relation to zinc remained unclear, Vallee and Margoshes concluded that their results were "indicative of a low molecular weight protein, probably containing a small number of cadmium atoms per molecule." (1957, p. 4813) [3] Though its biological function and significance remained completely unclear, this marks the discovery of metallothionein (MT).

It took until 1960 for the term "metallothionein" to be first coined [5]. The name is descriptive in nature. It points to a bond between a metal ion and thionein (a cysteine rich apoprotein) rather than describing a function of these kinds of proteins *per se* [6]. Though it was not immediately obvious what MTs can and cannot do, and what purpose they might serve in a living organism, MTs became the focus of an intense and often controversial research corpus since the 1960s (*e.g.* [7–12]). From a biochemical perspective, there is no question that MTs have unique properties. Besides, they are extremely widespread across biological organisms. Based on genetic information, 15 families of MTs have been identified in mammalian and nonmammalian animal species [13]; four further families have been identified in plant species [14]. Both their biochemical characteristics and their taxonomic prevalence speak to MTs serving some important biological role(s). Six decades after their initial discovery, after thousands of studies describing their structure, biochemical characteristics, and tissue distributions, however, the biological function(s) of MTs still remains enigmatic [15,16].

In this paper, we shall discuss both the discovery of as well as recent research on MTs from a philosophy of science point of view. We shall systematically analyze the kind of research that has been done on MTs and what new research strategies might be worthy of pursuit given the status quo. The paper is structured as follows: In section 2 we shall discuss what features Dr. Vallee's initial research on MTs exhibits, how these contrast with dominant research strategies in biochemistry as well as related disciplines such as pharmacology, and how to account for them within a philosophical framework of scientific inquiry. In section 3, we shall review more recent research on the MT family. We shall outline three specific hypotheses that have been pursued with respect to MTs' biological functions. Though searching for one-to-one molecule-function mappings has been successful in other cases, as we shall outline in section 4, this strategy has not yielded a satisfactory result for MTs to date. Against this backdrop, we shall suggest in section 5, it might be a worthwhile to consider other research strategies worthy of pursuit in order to understand the biological and evolutionary role(s) of MTs.

2. Classifying Vallee's Experiments

The dominant picture of research in biochemistry and related disciplines such as pharmacology is that scientists start with a function. They notice, say, that some plants have the ability to convert light into energy and wonder how that works. Or they get interested in how blood pressure is kept constant or muscle growth is being regulated by on organism. In all of these cases, the researchers start working from an explanandum (a phenomenon to be explained) to an explanation of how that phenomenon or function is elicited. Paradigmatically, much of the research needed here will be focused on decom-

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posing the system in question, looking at what the different parts of the system do precisely, and how they work together to elicit the explanandum. This analysis may take different forms and run through multiple operations but the essential rationale behind any version of this downward-looking approach might be captured by the slogan "divide and conquer!" – researchers are breaking up the system into units, and associating each unit with a specific function [17,18].

2.1. Downward-Looking vs. Upward-Looking

While downward-looking is a good strategy in many cases, this is not all there is to science and scientific inquiry. Recent philosophical accounts of discovery and explanation have been highlighting that identifying the mechanism responsible for a phenomenon to be explained may involve bottom-up or upward-looking research along with downward-looking decomposition strategies, Figure 1 [19–21]. That is to say, rather than just taking a system apart and studying what the different components (parts) do in isolation, researchers are frequently studying individual components while they are looking for what these components do as part of an overall system. If you want to understand, say, hippocampal long-term potentiation (LTP), it is not only interesting to see *that N*-methyl-D-aspartate (NMDA) receptors are part of the mechanism in question. You might want to know *how* NMDA receptors contribute to LTP—how they work, how they are triggered, and what happens to LTP if there are no NMDA receptors. So even if you are starting from an explanandum (LTP in this case), at some point you might need to employ upward-looking research focusing on how a component (the NMDA receptor) contributes to the explanandum.

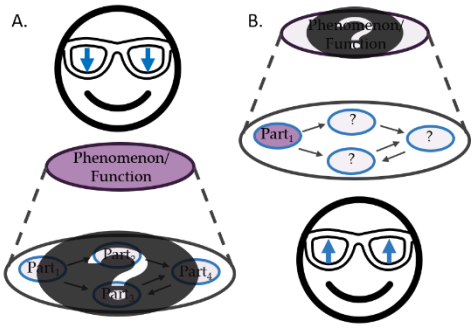

Figure 1. Ilustratining the difference between upward looking and downward looking approaches to research. Panel A, represents the downward looking approach, *e.g.* decomposition. Panel B, represents the upward looking approach, *e.g.* compostion or "re-composition".

To illustrate this downward-looking research strategy, we might also consider the case of penicillinase. Almost a decade after Alexander Fleming's discovery of penicillin in cell cultures of the Gram-positive *Staphylococcus aureus* contaminated by the fungus *Penicillium rubens*, attention was shifted to elucidate antibiotic resistance observed in some bacteria [22]. Reserachers prepared purified extracts from crushed penicillin resistant *Escherichia coli*, incubated them with penicillin to then introduce it to bacterial cultures known to be sensitive to penicillin [23]. The processed penicillin demonstrated a significantly lower antimicrobial effect than regular penicillin. When the same kind of extract was prepared from penicillin sensitive *Staphylococcus aureus* no such effect was observed. Researchers continued to look for the active substance that was responsible for this effect. In 1940, they identified a protein, termed penicillinase, which they believed to be responsible for inactivating penicillin [24]; further studies confirming the different sensitivity towards penicillin due to the absence or presence of penicillinase were conducted [25]. Put briefly, we might summarize the case as follows: Scientists started searching for what makes bacteria resistant to penicillin by downward-looking research. They crushed resistant bacteria and examined their components. Once they identified a

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likely candidate (in this case: penicillinase) responsible for the phenomenon (penicillin-resistance), they assessed its relevance in an upward-looking experiment by putting penicillinase into penicillin-sensitive bacteria.

What does this tell us with respect to the case of MTs? With the distinction between upward-looking and downward-looking experiments in place we can clearly recognize Vallee's early research on MTs (along with most recent MT research we will discuss below, in section 3), as upward-looking: it focuses on a molecule rather than a function we are trying to explain. This is intriguing. Not because upward-looking research is rare in biochemistry and related disciplines *per se*. But because in the case of MTs it was (and to a large extent still is) the starting point; MTs have not been identified by some sort of downward-looking study. It is noteworthy that not all MTs were the product of upward looking research. MT3, for instance, was identified after the observation that extracts from brain cells of Alzheimer's disease patients supported the survival of rat neuronal cell cultures and was initially called Growth Inhibitory Factor (GIF).Before we continue discussing the nature and prospects of upward-looking research, let us turn to two other distinctions that will be helpful in understanding what sets Vallee's discovery of MTs apart from much other biochemical research.

2.2. Interventions vs. Mere Interactions

Experimental work is often characterized in terms of interventions. While the notion is widely used in the empirical sciences to refer to any kind of manipulation, philosophers of science distinguish between different kinds of experimental strategies and manipulations. In this context, the notion "intervention" is reserved for those manipulations that systematically wiggle some factor X in order to elicit changes in another factor Y (cf. *e.g.* see [26,27]). Researchers may intervene (*i.e.* inhibit or trigger), for instance, into the activity of some part of a system (X) to study how that intervention affects the system's overall behavior (Y)—or *vice versa* [28]. We may study, say, how penicillinase affects penicillin-resistance or how damaged NMDA receptors interfere with LTP. Intervention-based experimentation is experimentation that manipulates something *in order to study the effects it elicits*.

Intervention-based research can be contrasted with merely interactive experimentation [29]. Mere interactions are a different form of experimental manipulation; they serve to uncover structures and organization and make accessible features of a system without changing these features. Cases in point are (among many others) the application of staining techniques, centrifuge of fluids, optogenetics, or—as in Vallee's case—spectroscopy. In any of these cases, the system is affected, and some aspects of its are being changed (*e.g.* by staining them). But while interventions are applied to study what effects certain manipulations will have on the system or its parts, mere interactions are *employed precisely because they have certain well-known effects* (such as attaching marker molecules to certain particles). Unlike interventions, mere interactions do not seek to interfere with what a system or any given part of it does. Rather, mere interactions are *sophisticated tools for observation* that help scientists uncover features of a system or organism not otherwise accessible.

With this distinction in place, we can recognize, that at least initially, Vallee's work on the kidneys of various species as a case of mere interactions. He did not seek to change the cadmium content of the tissues he studied to see what happens when cadmium content is raised or lowered. Rather, he measured it with the technology available to him. He also did not change the kind of protein cadmium was bound to but analyzed its biochemical properties through merely interactive experimentation. Note that while Vallee's initial mere interactions might be considered a sort of decomposition, they are not downward looking in the sense that he was searching for a component relevant to a specific function he sought to explain (see section 2.1 and Figure 1).

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2.3. Hypothesis-Testing vs. Exploratory Research

Interventions in the sense just described are frequently carried out to test specific research hypotheses. If we hypothesize that penicillinase plays an important role in penicillin-resistance, for instance, we can test that hypothesis by *intervening into*, *i.e.* changing, the presence of penicillinase and observing what effects that has on penicillin-resistance. This approach might be contrasted with exploratory research, *viz.* research that does not test hypotheses or theories. *Exploratory experiments* are often conducted to map the functional architecture of a system without having a pre-established theory, or to delineate phenomena from their surroundings [30–34]. Exploratory experimentation is often very popular before a scientific theory or research tradition gets established and before specific hypotheses can be formulated. While it seems tempting to associate exploratory research with mere interactions and contrast it with hypothesis-testing interventions, this dichotomy is premature. In fact, exploratory research can utilize interventions as well (just think of Hubel and Wiesel's systematic research on cat V1, or Charles Dufay's "discovery" of the two electricities (positive and negative) [35,36]. In the same way, hypothesis-testing research can utilize mere interactions (if, say, we are hypothesizing that a chemical we can stain is contained in a cell) [37].

To summarize the terminology just introduced: experiments can be (i) either exploratory or designed to test specific hypotheses, they can (ii) utilize interventions (studying the effects of a manipulation) or mere interactions (manipulations serving to uncover certain structural features), and they can (iii) be downward-looking (*i.e.* starting from the phenomenon and searching for what elicits it) or upward-looking (studying the contribution of a component to a phenomenon or a system's overall behavior). Without going into the philosophical details here, let us add that classifications with respect (i)-(iii) are independent of one another. Yet, it is worth pointing out that exploratory research and experiments utilizing mere-interactions tend to be more common at the beginning stages of a research process whereas intervention-based hypothesis-testing research tends to be more common when a research tradition has matured, and results are being published [37]. Against this background it is little surprising that the dominant view of biochemistry is not only that it focuses on downward-looking research but also that is utilizes interventions and hypothesis testing. After all, most research is being conducted and published once a research tradition has matured.

But, again, what do we learn from all this about MT research? First, we already noted that MT research is mostly an upward-looking search for a function of a biochemical component. And though this may not be the way biochemists typically identify what molecules serve which biological function, this can actually be a successful research strategy, as we will see in section 4. Second, we can now recognize that Vallee's work was exploratory in nature—he did not have pre-established theories and he was not working to test hypotheses—and that his methodology was based on mere interactions, such as spectroscopy. While these features, too, may seem intriguing if compared to the large body of published research in biochemistry, it is little surprising to find exploratory and mere interaction-based research where a molecule was first discovered. For this simply marks the beginning of a research tradition, in our case the hunt for elusive function of MTs [16]. As we shall see in the next section, MT research has developed into a more hypothesis-testing and intervention-based research tradition since the 1960s. But it remained very much focused on upward-looking research.

3. The Enigmatic Role of MTs

Since the initial discovery of MTs, biochemistry has made significant progress with respect to characterizing and classifying MTs. By now, we know that MTs are metalloproteins that have an exceptionally high sulfur content (concentrated in 20 cysteine residues) and can hence bind up to seven Zn^{2+} or Cd^{2+} ions or 12 Cu^{+} ions. The high negative charge of the unique metal-sulfur clusters, such as the Zn_4Cys_{11}-α-clusters and Zn_3Cys_9-β-clusters in MT-1 and MT-2, is balanced by seven lysine residues, resulting in

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the equally unique dumbbell shape of the MT proteins which sets them apart from many other biomolecules found in nature [38,39]. Although MTs have been identified in many organisms—many prokaryotes and almost every eukaryote carry genetic information encoding MTs—mammalian MTs are the ones most studied. In human cells, for instance, 14 genes encoding MTs reside on chromosome 16q13 and three different subfamilies based on location and phylogenic features have been identified, *i.e.*, MT1 and MT2 in the liver, kidney, spleen, MT3 in the nervous system and MT4 in the squamous epithelia [40–42]. Phylogenic analyses further categorize these subfamilies into subgroups, *i.e.*, m1P1 and m2P2 as subgroups of MT1 and MT2, respectively, in humans. From these subgroups several human isoforms are specified *e.g.*, MT1A and MT1B. Genetically, human MT1 and MT2 are much less discrete than MT3 and MT4. In fact, MT2 is a member of the MT1 branch, Figure 2 [13,43].

Despite this detailed knowledge about the structure and phylogenetic relations of MTs, researchers' knowledge of MT function remains very limited to date. Nevertheless, the widespread prevalence of MTs across organisms and their unique biochemical

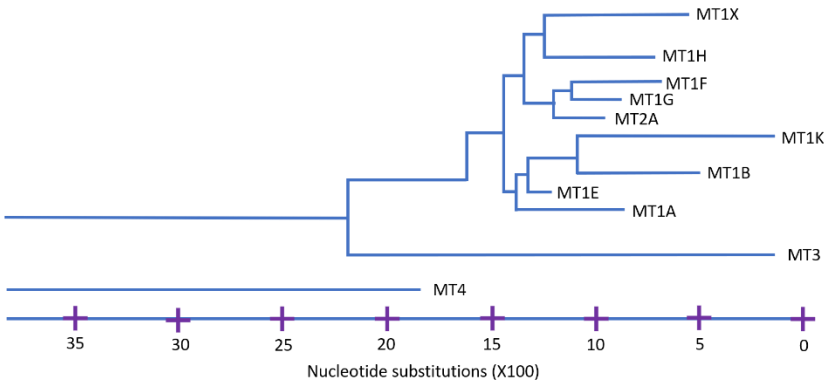

Figure 2. Phylogenetic tree of protein sequences of human. (Adapted from Laukens *et al* 2009 p.304)

properties suggest that MTs serve some important biological role. And indeed, several functions for MTs have been suggested: For a long time, research has focused on the reactivity and sensitivity of the cysteine ligands towards metalation, oxidation and reduction [12,44–46]. Besides, the absence or impairment of MTs has been linked to serious medical conditions such as pancreas, kidney, and liver damage due to excess amounts of free metal ions (Zn^{2+}, Cu^{+}, Cd^{2+} and Hg^{2+}) and heart tissue damage due to oxidative stress [47–49]. Hence, three main functions have been proposed over the past 60 years: metal detoxification or toxic metal metabolism, metal homeostasis or essential metal metabolism and oxidative stress protection or antioxidant. In what follows we shall briefly review these hypotheses and associated research.

3.1. Metal detoxification or toxic metal metabolism

As the first MTs were isolated from Cd-poisoned horses, the notion of MTs as detoxifying proteins for heavy metals has been postulated. MT knock out mice are observed to be more sensitive to tissue damage due to the administration of cadmium, in addition to other *in vivo* studies reporting an upregulation of MTs after such administration *[50–52]*. A free Cd^{2+} ion inside the cell displaces zinc from MTs leading to an increase in the intracellular concentration of free Zn^{2+}. The displaced Zn^{2+} ion is picked up by a zinc sensitive protein, *i.e.* metal response transcription factor 1 (MTF-1) which is then translocated to the nuclei of the cell, where it activates a transcription promoter, metal response element (MRE), hence an induction of thionein (apoprotein). The affinity of MTs to metal ions follows the binding constant of thiolates ($Hg^{2+} > Cu^{+} > Cd^{2+} > Zn^{2+}$). Inside the cell, however, MTs are mostly bound to either zinc or copper (two essential

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metals), thus MTs bound to cadmium and other heavy metals are believed to result from exposure to environmental factors *[53]*.

3.2. Metal homeostasis or essential metal metabolism

Native MTs isolated from mammalian cells are generally bound to zinc and copper ions. Both metals are metabolically important and their intracellular free ion concentrations are in the picomolar range, despite their micromolar total cell content. Zinc has catalytic, structural, and regulative roles in the biology and is a prosthetic group in about 3000 human proteins *[54]*. As opposed to a previous understanding of the metalation mechanism of MTs which was suggested to be of a cooperative nature, *i.e*, the binding of one zinc ion, for instance, would facilitate the binding of the next zinc ion and so on (this also suggests the absence or instability of partially metalated MTs within the cell), accumulating evidence demonstrate that MTs bind metal ions uncooperatively *[55]*. Such findings have important implications on our understanding on how MTs are involved in essential metals metabolism. MTs are no longer regarded as zinc storages or thermodynamic sinks, rather as an active component in both the regulative and cellular singling pathway of zinc tightly controlling its transient concentration as buffers.

3.3. Oxidative stress protection or antioxidant scavengers

The antioxidant function of MTs is proposed due to the exceptionally rich cysteine content (30%) and the fact that oxidative stress also leads to upregulation of these proteins [56]. *In vitro* and *in vivo* studies demonstrated that ZnMTs scavenged free radicals when treated with hydrogen peroxide and released Zn^{2+} which in turn led to MTs induction through MTF-1 and MRE. Reactive oxygen species (ROS) easily oxidize MTs leading to their cellular upregulation mediated by an antioxidant response element (ARE) activated by an ARE-binding transcription factor [57]. Increased expression of these proteins mitigates reperfusion injury in the muscle tissues of the heart [58]. It is also reported that MTs inhibit the copper induced hydroxyl radical.

3.4. Quo Vadis?

What do we learn from all of this? Put briefly: despite thousands of studies describing the structure, biochemical characteristics, and tissue distributions of MTs, and even specific suggestions for what role MTs might play in biological organisms, the actual function of the MT family still remains enigmatic [15,16]. We might wonder why that is and whether, maybe, there is some sort of systematic problem with MT research. Indeed, some authors have highlighted that MT research faces several non-trivial experimental difficulties [7,55]. For instance, applying X-ray diffraction has provided great insights into the dumbbell shaped crystalline structure of MTs. Yet such experiments are reported to face significant difficulties and hence, most of the structural characteristics are obtained through NMR analysis which illuminate the coordination of metal-thiolate ligands in the two functional domains but not *how* they connect and interact [59–61]. Another difficulty stems from the fact that d^{10} metals are chromophorically silent in spectroscopic essays. Hence, when studying metal ion transfer scientists substitute zinc with cobalt as a spectroscopic probe [62]. Moreover, the *in vitro* studies of MTs do not account for the *in vivo* heterogeneity of these proteins (varying amount and content of metal ions) and the structural models are derived from one isoform, *i.e.* Zn4Cys11-α-clusters and Zn3Cys9-β-clusters [7,15]. Due to such limitations, one might argue, it is difficult to make general claims about the function of MTs. But is this really a systematic weak point of MT research? Limited, indirect or suboptimal experimental methodologies and tools are common across the empirical sciences. And quite generally speaking, this does not prohibit progress in principle. Neuroscientists, for instance, study the brain with all kinds of epistemically limited tools. Still, we have a good idea about what certain structures and molecules in the brain do. To be fair, methodological limita-

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tions surely are not making it easier to identify MTs' functions. But that is hardly the sole reason for us not knowing what the function of MTs is.

If limited experimental methods are not the issue, one might suspect that identifying MT functions does not currently get off the ground due to the kinds of experiments used. Based on our exposition in section 2, however, we can conclude that the use of exploratory research and mere interactions is neither special nor problematic for an episode of scientific inquiry that lays the foundations of a new research tradition. And indeed, recent research—as we have just outlined—has moved well beyond this: there have been theories developed, hypotheses proposed and tested, interventions carried out, etc. So what is left then is the upward-looking character of the overall MT research tradition: if the prototypical approach in biochemistry is to start with a function and search for the molecule(s) relevant for that function, having a molecule and searching for its function might seem a bit like putting the cart before the horse. So the attentive reader may suspect this is where a potential systematic problem might arise. Though, as we shall show in the next section, it is not actually as simple as that either.

4. The Case of Ceruloplasmin

To see why upward-looking research is not in principle unable to pinpoint a molecule's function, consider hemoglobin. Hemoglobin is an iron metalloprotein present in the blood cells of almost all vertebras and some invertebrates. It was discovered the first time in samples of earthworm blood by Friedrich Ludwig Hünefeld (1799 – 1882) in 1840 **[63,64]**. While its function was initially unknown, 30 years later French physiologist Claude Bernard (1813 – 1878) **[65]** recognized hemoglobin's oxygen carrying property.

For a more recent example, consider the case of ceruloplasmin. In 1944, Carl G. Holmberg (1906 – 1991) reported the observation of a unique blue coloration in his insoluble serum fractionation sample, P_I-fraction **[66]**. The blue color reminded him of the inactive copper protein isolated by Mann and Keilin in 1938 from the red blood cells of equine serum, named haemocuprein (later to be known as superoxide dismutase (SOD)), inspiring him to identify the copper content of P_I-fraction **[66]**. He quickly realized that this small fraction of the serum contained "proportionally about five times so much copper as the serum protein as a whole."(**[66]** p.228) Holmberg also noted that the blue color can reversibly be oxidized and reduced, hinting towards an enzymatic activity. Today, ceruloplasmin is known to play an important role in iron metabolism and be the main storage for copper in the human body (accounting for 95% of the total body content) **[67,68]**.

Both these cases clearly illustrate that searching for a molecule's functions (at least sometimes) is successful in biochemistry. Put slightly differently, that is to say upward-looking research programs are not in principle doomed or systematically flawed. They are viable and can indeed be successful. Thus, that the function of MTs has not yet been identified cannot be blamed on the fact that the search started from the molecule. If this is correct, the reader may wonder, what is left? How come we still do not know the function of MTs? In the next section, we shall offer a few other considerations that might be helpful to advance our understanding of the enigmatic MTs.

5. Beyond One-to-One Mappings?

From a philosophy of science point of view, searching for "the" one function of a biochemical molecule seems highly idealized. And even if upward-looking research has been successful to associate other molecules with a specific function, that is no guarantee that this strategy will work for the MT family. For sure, all of the suggested functions for MTs have seemed worthy to pursue at some time (cf. section 3). But given that there is not much progress being made based on the assumption that MTs serve some sort of unitary evolved biological function across organisms, we might have to change tack. In what follows, we propose two ways of thinking that might lead to new research agendas.

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5.1 Multiple Realization, Compensation, and Moonlighting

The first set of considerations we would like to offer revolves around the idea that one-to-one mappings between biochemical components and biological functions might not be realistic. For instance, MTs might serve an important biological function but that function might not actually be unique to MTs. What MTs do in biological organisms might also be achieved by other biochemical molecules. To see where our thoughts are headed, consider the case of hemophilia. Hemophilia is a devastating medical condition characterized by a malfunctioning clotting mechanism, resulting in a potentially life-threatening extended hemorrhage period. At least three types of hemophilia have been identified, hemophilia A, B and C, each corresponding to deficiencies in a different clotting factor either genetically or due to autoimmune diseases, cancer, or pregnancy. For a long time, the distinction between the three types went unnoticed [69]. In 1952, Christmas disease (now known as hemophilia B) was suggested [70]. The researchers started from the observations that adding small amounts of normal blood to blood samples from hemophilic patients reduces the clotting time and that in some cases a similar time reduction is observed when mixing two hemophilic samples from different patients. When isolated antihematophilic globulin (now known as factor VIII), was added to some of the hemophilic samples no reduction in clotting time was observed, hence, indicating a variation in the underlying clotting factor (component of the mechanism of clotting) responsible for the prolonged clotting period (Christmas disease). Further fractionation and precipitation of the blood samples from the Christmas disease patients demonstrated an absence of a specific protein, when compared to normal blood, and hemophilic samples (lacking factor VIII a characteristic of hemophilia A). This active protein was called thereafter, Christmas factor or factor IX.

What is noteworthy about hemophilia is that there is not just one biochemical factor eliciting it, but three different ones. Thus, we see a mapping from one phenomenon (a biological function) to many molecules exhibiting that function (we might consider this a case of *multiple realization*). Now if we assume that this is the case for MTs, too, we would basically have to expect that MTs serve as one of multiple realizers for one or many functions. If so, this might explain why upward-looking research is actually at its limit to understand the role that MTs play in biological organisms; for identifying different biochemical components relevant to a single phenomenon requires knowing what the phenomenon in question is and carrying out downward-looking research. To this end, all the upward-looking MT research done to date will provide useful clues, but it simply will not be conclusive.

Another, though related, reason why it might be so difficult to pin down MTs' biological function could be that their function might be easily compensated for by other biochemical components or mechanisms if MTs are missing. Or, conversely, MTs might themselves serve as a compensatory function in case other important structures or mechanisms fail to operate. The existence of such compensatory mechanisms is at least quite plausible. After all, we know that redundancy and plasticity are important principles to make organisms fit for survival. For instance, consider connexins (Cx) the transmembrane proteins which allows the exchange of small molecules between neighboring cells [71]. Cx36 compensates to the function of Cx45 in the retina and Cx32 was reported to compensate for Cx43 [72,73]. But if this hypothesis is correct, the question remains as to how MT researchers might work their way through the tickets of metabolism in various species to understand the precise role of the MT family.

A third, and perhaps the most likely option is that although MTs form a seemingly united family of proteins they actually serve a host of different functions in various biological organisms. That is to say, we have a single biochemical molecule that does multiple things in a biological organism. For illustration consider the case of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In glycolysis, GAPDH serves an enzymatic function catalyzing the sixth step of the reaction, *i.e.* the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate. GAPDH has been functionally linked to

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several nonmetabolic functions such as initiation of cell death, transcription of DNA, participation in the antioxidant defense mechanism and metal chaperoning [74–77].

If something like this is the case for MTs, too, one might wonder where the multifunctionality of MTs might come from. Recent discussions about protein moonlighting [78,79] suggest an answer: biochemical molecules can actually acquire new and diverse functions through evolution. Proteins can have moonlighting functions based on their localization intracellularly or extracellularly, the types of cells expressing them, the type of substrate available, their quaternary structure (monomers or multimers), or their interaction with other proteins to form more complex structures and multiplicity of binding sites [80]. Against this background, some sort of accidental or systematic biochemical resemblance despite diverse functionality seems at least plausible—and perhaps more feasible than to expect a one-to-one mapping between biological functions and biochemical molecules. If this is correct, searching for "the function" of MTs might not be a productive research strategy. We might be better off asking where the MT family comes from and how it has developed.

5.2 Evolutionary Effects: Vestiges, Exaptation, Convergent Evolution

The second set of considerations we would like to offer originates from the following question: What if the answer to the question of why MTs are so widespread does not lie in their current but in a *past* biological function? In other words, we would like to point to some evolutionary considerations.

First, we might wonder whether MTs could be vestiges of some sort. That is to say, could they be residues from our evolutionary ancestry that no longer have a biological function? Given how widespread MTs are throughout the animal and plant kingdom, however, MTs would have to go back very far and, there would have to be a common root in the phylogenetic tree of MTs which does not seem to exist (cf. section 3). An example of a vestige in humans would be the non-functional gene encoding *L*-gulonolactone oxidase required for the biosynthesis of ascorbic acid [81].

In light of this, we might consider another option: the MT family might actually be a result of convergent evolution. That is to say the same kind of biochemical molecule evolved in different species to perform different, though possibly related, functions. Maybe all of these functions are somehow linked to metabolizing metals and that is what makes the sulfur-rich metalloproteins such a good candidate molecule for the jobs in question. Alternatively, MTs might have had some crucial shared biological function that has shifted throughout evolution (exaptation). As a result, we may still see a family of MTs united by a shared biochemistry that now serves different functions in different species or organisms. This is perhaps the most promising among our evolutionary considerations: it is effectively a version of the many-functions hypothesis outlined above. Since it is compatible with moonlighting, it comes with a candidate mechanism for how multiple functions have been acquired by the MT family. And as such, it comes with an evolutionary explanation for the shared biochemistry despite varied functionality within the MT family. It does not require a common root in the phylogenetic tree because it does not require all MTs to have some sort of common ancestor while also being compatible with the idea that all MTs did have some sort of unitary function at some point.

Note that all of the hypotheses just sketched come with a modification of the central research strategy in the investigation of MTs' function(s). Research on MTs has initially started from the recognition that they are extremely prevalent across the animal and plant kingdoms. "Why do so many species have MTs?" was thus interpreted as a question about MTs' *function*, demanding researchers to find out *what MTs are good for*. But having an important biological function today is not the only possible reason for why some biochemical molecule may be widespread. The hypotheses we outlined in this section acknowledge that and we suggest that—rather than searching for the function of MTs—we should wonder about possible *origins* of MTs to explain their prevalence. Of course, our considerations are primarily theoretical in character. Whether any of the hypotheses we suggest here are actually worth entertaining will, of course, be up to prac-

ticing scientists in the field to decide. But from a theoretical point of view, they seem at least interesting and they are backed by philosophical considerations of pursuitworthiness and scientific progress (*e.g.* [82,83])

6. Conclusions

None of the ideas we put forward in the previous section are devised theories of MT function. Yet, they might offer useful hypotheses for experts in the field to pursue. What we can conclude at this point is the following: Despite the special circumstances of the discovery of MTs, there seems to be nothing systematically amiss in the research tradition. What appears so intriguing about Vallee's early work is actually characteristic of discovery episodes leading to the foundation of a research tradition: exploratory research involving mere interactions. Besides, the upward-looking search for MT functions is—at least in principle—a viable strategy that has been successful in some cases (see section 4).

Yet it looks like MT research has not been going forward as much as biochemists would have liked it to and the biological role(s) of MTs still remain enigmatic. Thus, it might be a good idea to look at strategies, theories and hypotheses that have not yet been pursued—and section 5 might be taken to offer suggestions. At the very least, it seems worth looking into new research hypotheses to examine whether any of them might be worth pursuing. And if they are, that might help to finally elucidate why MTs are so prevalent, what precisely they do, and where they come from.

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