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Crystallizing Techniques: Sample Preparations, Technical Knowledge, and the Characterization of Blood Crystals, 1840-1909

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ABSTRACT: Sample preparation is the process of altering a naturally occurring object into a representative form that is amenable to scientific inquiry. Preparation is an important preliminary to data collection, ubiquitous in the life sciences and elsewhere, yet relatively neglected in historical and philosophical literature. This paper presents a detailed historical case study involving the preparation and study of blood crystals in the nineteenth century. The case is used to highlight significant features of preparation, which aid our understanding of the epistemology of sciences in which preparations play an important role. First, it shows the role of technical knowledge in efforts to characterize a scientific phenomenon or object of interest. Especially in early stages of characterization, scientists improve their understanding of what they are preparing by better understanding their preparation procedures. Second, this case contributes to recent views of characterization as a relatively autonomous domain of scientific activity. It shows how preparation functions as a site for integrating different experimental methods, and the difficulties that ensue. In light of these considerations, the case shows how characterization is capable of shaping or constraining explanatory pursuits as much as it is guided by them.

1 Introduction

Effectively studying an object of inquiry requires that it undergo some form of preparation for study. The object and instruments with which it interacts must be composed into a suitable arrangement for generating data. Preparations typically precede data collection, though the quality of downstream data

depends crucially on them. This makes preparation a crucial element of scientific experimentation. Sample preparation is widespread in chemistry, biology, and other historical sciences. Prepared samples are often designed to enable multiple forms of intervention and investigation; they are key nodes in the multi-instrumental integrative approaches found in many special sciences, frequently arising as the "boundary objects" between contiguous sub-fields and groups of knowers (Star & Griesemer, 1989; Bowker & Star, 1999; Bowker, Timmermans, Clarke, & Balka, 2015). Understanding the production and uses of sample preparations is thus of plain interest to the epistemology of science.

Preparation is also a longstanding category in international scientific vocabulary. The historical texts I will discuss routinely describe the series of manipulations that produce an object of study as a "préparation" (Berzelius, 1813, p. 70), they separate sections labeled "Preparation" from others like "Tests" (Lehmann, 1855), they discuss diverse methods of preparing as "Präparationsmethoden" (Funke, 1876, p. 230), and distinguish "methods for preparing, examining, and measuring" an object of study (Reichert & Brown, 1909, p. 141). For all that, preparation has not been a subject of sustained historical and philosophical attention.[1] One aim of this paper is to correct this oversight and foreground the epistemic significance of preparations in science. A first step in this direction has been taken by Rheinberger (2010, pp. 233-43). He notes that preparations, despite resulting from a series of manipulations, have scientific value because they are made of the same material as the natural objects for which they stand. Somewhat paradoxically, differences from the unprepared object in situ are only introduced so the preparation can be a better, more representative version of this object. In this respect, preparations might reasonably be thought to raise issues in common with philosophical and historical work on model organisms (Kohler, 1994; Ankeny & Leonelli, 2021). However, whereas many model organisms in contemporary science can be acquired readymade, samples need to be prepared again and again.[2] Focusing on prepared samples helps foreground the epistemic role of preparation procedures—how an object is reworked to enhance understanding of that same object or a related phenomenon.

[1] This is especially true of philosophical literature. In history, shared themes are found, e.g., in Landecker (2007).
[2] Thanks to an anonymous reviewer for highlighting this contrast.

How do preparations achieve such representative status? Rheinberger elicits this question but doesn't answer it. This paper will address the question by considering the historical development of a form of preparation that has persisted over nearly two centuries of modern science: the crystallization of biological materials. To this day its application to a new protein, virus, or other macromolecule is uncertain and potentially laborious; references to crystallization as an art lacking universal method are found in contemporary reviews (Giegé, 2013). Nevertheless, researchers have strategies for handling their samples. The crystallization of animal blood provides an early case of how these develop.

The history of blood crystallization highlights several features of the epistemic role of preparations in the sciences in which they predominate. First, it shows that scientists come to characterize the object of inquiry that their preparations stand for through an understanding of their preparation techniques. Especially at the early stages of investigation, this technical knowledge is a vital bridge between data and phenomenon. Second, it shows how this technical knowledge can develop and persist with a degree of autonomy from specific data-gathering or explanatory aims. This autonomy, in turn, shows why preparations are productive sites for the integration of diverse forms of experiment in the sciences. Methods of data generation applied to preparations allow for enriched characterizations of the prepared object of inquiry, albeit in ways that may channel or constrain explanatory pursuits. I will elaborate on each of these points before turning to historical details.

Prepared samples function as sources of data. Scientists use these data to infer the characteristics of objects or phenomena of inquiry.[3] Preparations thus play a foundational role in the *characterization* of scientific objects. For data sourced from a preparation to provide reliable inferences about an object of study, a preparation must bring out something "already there." This, in turn, depends on a proper understanding of what one does to a sample in preparing it. Properly characterizing an object of inquiry from prepared samples thus depends on some preconception of this object and an understanding of preparation itself. Hence, a first claim supported by the blood crystal case is that preparations are a significant site at which the objective and practical domains of scientific knowledge become intertwined.

[3] For data-to-phenomenon inference, see (Bogen & Woodward, 1988).

Researchers come to understand what their sample stands for through an understanding of what is done to it in the preparation process. This is borne out by my case study: by scrutinizing individual steps in blood crystallization protocols, scientists improved their ability to grow crystals and their understanding of just what these crystals were sampling. Scientists simultaneously converged on a conception of the contents of blood crystals as haemoglobin and on a stable preparation protocol for these same crystals.

A second, related point of the paper is to show how preparations "have a life of their own" and can thereby contribute to the integrative character of scientific research. Preparations highlight the ramified and scaffolding structure of experimental practices. They are stable, material products of prior interventions and the targets of subsequent interventions from which data are produced. The technical and procedural knowledge embodied in preparation precedes many forms of data collection.[4] It follows that preparations can be investigated through different interventional techniques, where data from each may be used to complement or correct the other. My case shows how, as blood crystallization procedures took on a consistent form, they enabled multiple lines of data production. A range of differences in crystal preparations were identified—chemical, morphological, and spectrographic. Comparing these data aided scientists' understanding of their preparation procedures, sharpened their judgments of crystal purity, and enabled classification of new types of crystal contents. At the same time, they presented a problem: researchers struggled to explain the variations found in different kinds of data in a unified way. Attempts to attribute these varieties to differences in either haemoglobin's chemical structure or phylogenetic relations between taxa were unsuccessful. Empirical differences among blood crystals ultimately raised questions that available investigative resources struggled to address, contributing to crystallization losing favor among biochemists in the early twentieth century. From one perspective, this looks like a scientific failure in blood crystal research. But such a judgment prioritizes the role of theoretical explanations in

[4] My notion of data differs slightly from Leonelli's (2015). Her definition of data—material artifacts rendered portable and having the potential to serve as evidence—applies to many prepared samples. I prefer to distinguish the material artifacts experimentally intervened upon from the separable records of those interactions, which serve as the evidential basis for claims about phenomena. Only the latter are data in my usage.

science and neglects the important body of knowledge formed around the preparation and manipulation of crystals, a practice that was revived intact when researchers found new uses for crystalline samples.

As Waters (2004; 2019) has argued, appreciating such technical knowledge as a constitutive element of scientific knowledge can shift our evaluation of science's success and failures. In particular, it draws our attention to the objects of scientific inquiry as not only the explananda of theories, but also as material things that scientists must learn to consistently produce, to manipulate, to transfer between diverse methods of data generation, and thus know in a deeply practical way—activities exemplified by preparation.[5] This shift in perspective accords with developing work in philosophy of science that investigates how scientists arrive at specific characterizations of the targets of explanation and modeling (Colaço, 2018; 2020; Elliot-Graves, 2020). Preparation, I noted, is an integral aspect of the experimental characterization of objects in many sciences. The historical case of blood crystallization contributes to recent arguments that characterization deserves further attention as a scientific practice that is not merely subordinate to explanatory pursuits within a single field (Dresow & Love, forthcoming). The activities involved in characterization may develop in a way that ties together multiple lines of data production, without reference to a sole explanatory aim. Finally, the case illustrates how practices of characterization can also direct researchers to certain kinds of data-gathering strategies and affect which research questions are made salient in this process. Characterization thereby shapes explanatory strategies as much as it is guided by them.[6]

2 Disciplinary contexts

[5] Baird (2004) provides an extended defense of these ways of working with material—and the materiality of technological objects themselves—as a form of knowledge neglected by traditional propositional and conceptual accounts of scientific knowledge.

[6] The case study recounted here focuses on the development of crystallization protocols alongside the interventional and inferential uses of the resulting crystals. It aligns with historical works that take constructs such as "investigative pathways" (Holmes, 1985) and "second-order concerns" (Schickore, 2007) in science as their primary units of analysis. While I focus on the material and practical dimensions of science, I am concerned with the role these play in the internal development of scientific understanding and leave out the impact of broader social and cultural factors. The analysis thus might be said to sit somewhere between the post-positivist and sociological approaches to the history of chemistry (McEvoy, 2010).

The early study of blood crystals began with a discovery by Friedrich Hünefeld in 1840 and culminated in a voluminous work by Edward Reichert and Amos Brown published in 1909. This research was situated at the intersection of two branches of nineteenth century chemistry: mineralogy, which encompassed studies of crystalline substances, and physiological chemistry.

Crystals of a given composition have characteristic angles which remain constant between their faces. This fact, well-known by the late 1700s, provided a means of classifying mineral types, aided by the contact goniometer. Contact goniometers combine two straightedges with a scaled semi-circle, allowing a user to read off angles when each straightedge is aligned with adjacent crystal faces (Fig. 1). Crystal growth was known to be uneven, perturbed by many ambient conditions. To correct this, the Parisian priest and naturalist René Haüy experimented with cutting crystalline substances along various axes. He proposed that the characteristic "primitive" form of each substance could be arrived at by repeatedly dividing crystals along those planes where they cleave most easily until no further division was possible. All other forms were deemed "secondary." Crystallography's goal, Haüy announced, was "to determine the form of the constituent molecules of crystals, and the manner in which they are arranged among each other within each crystal. I call this combination *structure*" (1784, p. 9).

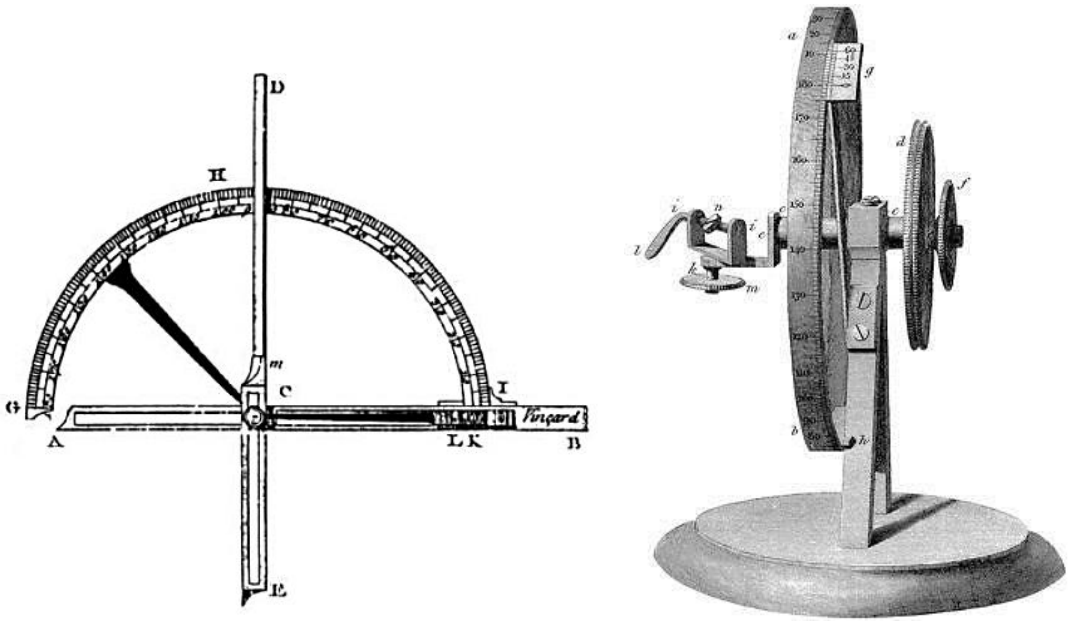


Figure 1: Left: a contact goniometer based on Carangeot's 1782 design, from Haüy (1822). Right: the first reflecting goniometer design from Wollaston (1809), in which an image is reflected off a face of a crystal fixed to horizontal plane. This plane is rotated by the vertical wheels until the image is seen once more in the crystal, at which point the angle of rotation is noted.

Haüy's "molecules" did not refer to atomic structures, but theoretical uniform building blocks that join at their faces to comprise a crystalline form. He hypothesized that crystals were built from regular, repeating units whose nature was systematically related to the shape and angles of macroscopic crystals. Haüy's goal was to establish laws of growth for each crystal by inferring the structure of its constituent molecules from a primitive form. In subsequent empirical work, such as the Oxford chemist John Kidd's *Outline of Mineralogy* (1809), crystals were grouped into classes based on their primitive polyhedral form, and further specified in terms of characteristic interfacial angles, hardness, and other empirical traits. Later works further classified crystals using qualities such as their refractive and color-changing effects on polarized light (Brewster, 1831). By the mid-nineteenth century, scientists seeking to identify a crystal had arsenals of empirical techniques and classificatory guidebooks at hand.

Unlike the crystals familiar to mineralogists, blood crystals were derived from biological substances. Growing them required skills more common to the fields of organic and physiological chemistry. Though practiced across Europe and America, this field was dominated by German chemists in the mid-nineteenth century, aided by state investment in burgeoning research universities. State interest in

physiological chemistry at this time was exemplified by the development of agricultural "experiment stations" in which researchers studied topics like the impact of different nutrients on livestock health. Major figures in mid-nineteenth century German chemistry such as Gerardus Müller, Justus Liebig, and Karl Lehmann were employed in such stations (Finlay, 1992).

Novel methods for identifying and quantitatively analyzing organic compounds brought about by scientists like Claude Bernard and Liebig stoked interest in the chemical study of vital phenomena such as respiration, digestion, and fermentation (Holmes, 1974). Inspired by Antoine Lavoisier's pioneering decompositions of water (1783) and airs, practitioners transferred the concept of chemical elements to living things. Animal substances were thought to be composed of "remote parts" such as fluids, oils, acids, and coloring matter (Brooks, 1803). The role of the chemist was to decompose these substances or otherwise find methods for detecting their constituents (Driggers, 2023). Some chemical reactions, once understood, became empirical tests for identifying components of interest. For example, by the 1850s three "color tests" existed for proteins, all based on the observation of a specific hue when a protein-containing substance was treated with a particular acid solution. Development in this field often took the form of an accumulation of protocols: new steps for isolating substances, testing for their presence, and otherwise producing observable effects. By this means, chemists working with organic materials followed the broadly classificatory approach found in concurrent mineralogical and crystallographic texts, seeking to characterize substances by clusters of empirical qualities.[7] The labs and instrumentation of these chemists do not look profoundly different from those used by 20th century biochemists. As with mineralogy, advances were primarily in the design and precision of equipment. Some significant novelties were introduced, such as Bunsen's spectroscope and gas burner, but the basic operations often remained the same: heating, cooling, mixing, precipitating, filtering, decanting, evaporating, and the like, all performed with customized glassware. Many shifts in the field instead occurred at the level of procedures and in their accompanying descriptions.

[7] Classifications were less stable for plant and animal substances. Early attempts were based on their composition out of "primitive substances" that were arbitrarily chosen based on chemists' abilities to isolate them (Klein, 2003).

In the mid-1800s, German scientists regarded physiological and medical chemistry as applied work and hence less prestigious. Still, several leading chemists made their marks on the field. German work was foundational in training early generations of American and British biochemists. Some examples will illustrate this pedigree:[8] In 1841, Liebig left the field of pure organic chemistry to study physiology. He trained Adolph Strecker in Giessen, who then received a position in Tübingen overseeing the work of Felix Hoppe (later Hoppe-Seyler). Hoppe was a Virchow student from Berlin who wrote a foundational textbook and pioneered a major journal in the field. While in Berlin, Hoppe had trained Ernst Salkowski, who eventually became head of an American department of medicinal chemistry and mentored figures like Otto Folin. Liebig's longtime collaborator Friedrich Wöhler supervised Wilhelm Kühne, who went on to succeed Helmholtz in Heidelberg. There Kühne trained several founders of American biochemical research, including R. H. Chittenden (of Yale and Columbia) and J. J. Abel (of Michigan and Johns Hopkins, founder of the *Journal of Biological Chemistry*). Likewise for Britain: Michael Foster, a driving force behind the Cambridge School of Physiology, sent his pupils J. Langely, W. Gaskell, and A. S. Lea to study under Kühne. Ernest Starling at University College London, the "fountainhead of British physiology" (Kohler, 2008, p. 42), had also spent time in Kühne's lab.

Upstream links to German education can be found for almost every eminent figure and research institution in early Anglo biochemistry. German chemical knowledge and methods were the basis for the emerging physiological and biochemical schools of the Anglophone world, which would become the center of twentieth-century protein crystallography.[9] The development of techniques revolving around the analysis of blood crystals exemplify this trend; the first dedicated study of these substances was undertaken in 1840s Leipzig; the last covered here took place in Philadelphia in the early 1900s.

[8] The following prosopography draws on Kohler (2008).

[9] Research still existed in these regions prior to heavy German influence. Atwater (1978) describes pockets of the United States where pioneering physiological research was carried out before 1870, most prominently in University of Pennsylvania medical studies. Americans known for physiology in the mid-nineteenth century, such as Silas Weir Mitchell and John Call Dalton (arguably the first full-time US professor of physiology), studied under Claude Bernard in Paris, rather than Germans. However, they worked against a background of scarce funding and undeveloped educational institutes, which lasted into the century's last decades.

3 The investigation of blood crystals

3.1 The first studies of blood crystals

As Holmes (1985) has argued, Lavoisier's work in general chemistry evolved in constant dialogue with his research on physiological processes like respiration such that his works in each area can be viewed as a unified investigative project.[10] Among other phenomena, Lavoisier and his contemporaries were motivated by a perceived link between the vital function of respiration and color changes in blood exposed to air. Ensuing experiments by authors such as Tiedemann, Gmelin, Bertuch, and Magnus made clear that gaseous content could be extracted from blood. The precise way gases were contained in liquid became a point of dispute. Various forms of inquiry into the relation between blood, oxygen, and carbonic acid were carried out in the ensuing years.[11] This work pushed researchers to obtain a clearer understanding of the constituents of blood, so to better infer its functional properties. In an early chemical decomposition, Berzelius (1813) focused on three components: fibrin, albumin, and the "coloring matter" (*matière colorante*, similarly referred to in German texts as *Blutfarbstoff*, later *Blutroth*). Each reacted similarly to various interventions—coagulable like egg white, soluble in water, but not alcohol or ether; producing a yellow color upon treatment with nitric acid. These "albuminous bodies," Berzelius speculated, might be a modification of the same basic substance: "The largest chemical difference that one finds between fibrin, albumin, and coloring matter consists in a certain quantity of iron oxide contained in the coloring matter" (p. 45). In 1838, Mulder published a method for analyzing the content of fibrin, albumin, and casein (Mulder, 1838). Similar proportions of elements and atomic weights for fibrin and albumin suggested they were variants of one substance. Berzelius and Mulder called them "proteins."

At first, Berzelius's "coloring matter" was distinguished from proteins. Le Canu had claimed in 1830 that perceived commonalities were due to failures to separate it from blood albumin. Upon boiling the substance in acidified alcohol and removing the residue, Le Canu obtained a purified coloring matter that contained iron but failed to exhibit the properties observed in 1813. Repeating this method, Berzelius

[10] For two significant works on respiration see Lavoisier (1777) and his work with Laplace (1780), though further experiments with collaborators like Seguin continued until his execution.
[11] For historical accounts see Holmes (1995) and Edsall (1972).

chose the name haematin for this substance. He corrected Le Canu on the accompanying residue: it was not albumin, but something distinct, which he dubbed globulin. Mulder carried out analyses of each and confirmed that globulin was akin to the other proteins. By 1840 it was believed that coloring matter, which Berzelius had taken to calling *Blutroth*, was a mixture of the protein-like globulin and the iron-containing haematin.

The first recorded production of blood crystals occurred at this time. In a treatise on animal chemistry, Friedrich Hünefeld noted a curious result after leaving human and pig blood to dry between two glass plates: "I saw tabular crystalline precipitations appear, which looked sharply defined and bright red under the microscope" (1840, p. 160). Hünefeld took little interest beyond observing their dissolution in water, blood serum, and whole blood. The crystals were largely forgotten until 1851 when Otto Funke, a student in Lehmann's lab in Leipzig, published a study on blood taken from a horse's spleen. He reported "one of the strangest phenomena […] which to my knowledge has not yet been seen by anyone" (1851, p. 184).[12] Like Hünefeld, Funke had placed the blood between glass and added a small amount of water. As it evaporated, crystals formed. He varied his methods, noting that alcohol and ether could alternate as "mother liquors." Using a microscopic goniometer, Funke measured their interfacial angles and concluded all crystals were rhombic in form. He was otherwise "not yet in a position to publish anything more precise about their nature and chemical constitution" (p. 185).

Questions over their nature occupied Lehmann and Funke for two years. They found that blood from any part of the body could crystallize and that different source animals yielded different crystals, results summarized in the second edition of Lehmann's textbook, *Physiological Chemistry*.[13] Along with varying the mother liquor and animal, they experimented with ambient conditions, claiming that sunlight and exposure to oxygen, though not strictly necessary, "constitute the most essential conditions toward the

[12] Hünefeld's crystallization was not widely cited, though Lehmann knew his work. In 1861, Hoppe and others referred to "Funke's crystals." Funke's 1863 *Textbook of Physiological Chemistry* makes no reference to Hünefeld, though it acknowledges discoveries by the chemists B. Reichert, Leydig, and Kölliker. Funke said they "had seen blood crystals by chance, but not recognized their true nature" (p. 25). Leydig had in fact wondered in 1849 whether these were haematin crystals but went no further. The earliest credit of Hünefeld I found is in Preyer (1871).
[13] Published in 1853, first edition 1841. References are to the 1855 English translation.

rapid formation of these crystals" (1855, p. 349). Conversely, microscopic inspection showed the presence of fibrin inhibited growth, recommending fibrin removal as a preliminary step in crystal preparation. The crystals were said to come in three morphological types: prisms (human, most mammals, fish); tetrahedra (some rodents); and hexagonal tablets (only squirrels), each rendered in illustrated detail. Modifications in the growing method were required based on the source animal.

After preparation, Lehmann treated crystals with various chemicals and recorded the effects on form and coloration. He also noted obstacles to inferring the nature of their contents: "The discovery of a crystallizable protein-substance appeared at once to afford a new means for obtaining more secure points of support for the establishment of its true constitution; but hitherto the elementary analyses of this substance have not furnished the desired information" (p. 347). One reason for this lack of "any satisfactory amount of exactness" (p. 348) was suspected impurities in the crystals; separating crystal contents from cell membranes (stroma) proved difficult. Yet Lehmann was confident enough to assert, contra Berzelius, that "this crystalline substance is not a mixture of a pigment [i.e., haematin] and a protein-body, but a pure chemical compound," the decomposition of which is "the principal object to be had" (pp. 351-353). He called this compound haematocrystallin. While it "might naturally be supposed that the investigation of this subject would enable us to decide the much-disputed question of the interchange of gases in the circulating blood, […] I have hitherto been unable to obtain any reliable results from my own quantitative determinations" (p. 352). Elsewhere, Lehmann notes that haematin may participate in this process, but warns against identifying it with "the true coloring matter of the blood" (p. 267) due to difficulties in isolating it from globulin in its soluble (in vivo) state.

3.2 The contents and varieties of blood crystals

Funke and Lehmann cleared a path for dedicated research on blood crystals. Uncertainties over crystal contents and their biological significance were not easily resolved: What was the relation between the red crystals and the coloring matter *Blutroth*, the combination of haematin and globulin that Berzelius had extracted from liquid blood? And why did crystal forms vary from one species to the next? As questions

lingered, researchers explored techniques for consistently producing crystals amenable to microscopic and chemical analysis. This meant crystals that were easy to make, large, and numerous. Silas Weir Mitchell's "Observations on the Blood Crystals of the Sturgeon" (1858) illustrates this goal. Mitchell recommended the use of sturgeon rather than human blood for its readiness to crystallize. He cited Funke's method, then offered another: leave fish blood in an open container, exposed to light and temperatures of 60-70 degrees Fahrenheit for at least two days, then take a drop of decomposed substance and let it slightly evaporate without added water. Shortly "the most beautiful crystals […] form abundantly in the mass of blood, and may be easily examined" (p. 2).

Like Lehmann, Mitchell chemically treated prepared crystals and described the effects, primarily on color. Mitchell's observations diverged from Lehmann's at several points. The crystals he identified as "true albuminoid crystals" were hexagonal in shape, whereas Lehmann only found prisms in fish blood. Where Lehmann thought some coloring matter was an essential constituent of blood crystals, Mitchell claimed he could remove their redness with alcohol or by dissolution and recrystallization. He also claimed to see the same hexagonal form in crystals grown from a range of human bloods (varying over sex, disease states, even drawing from a fetus and placenta). This implied crystal forms were constant for a species, yet "Professor Johnston, of Baltimore, informs me as a contrast to this statement that the splenic vein blood of the opossum afford tetrahedral forms, whilst all the other blood of this animal yields rhombic crystals" (p. 3).

These variations troubled Felix Hoppe in Prussia. He was harboring doubts about the status of Lehmann's "haematocrystallin," said to exist in all vertebrates. How could the same compound be responsible for the variety of crystal forms grown from different animals' blood? Contemporary with Mitchell, Hoppe had begun to study the chemical basis of color changes in blood upon exposure to gas, first investigating *Blutroth*'s reaction to carbon monoxide. Hoppe had moved to Tübingen in 1861. There, Bunsen and Kirchoff had recently used gas burners to demonstrate characteristic spectra of light emitted from purified substances heated to high temperature. These complemented the substance's spectrometric absorption lines recorded on exposure to light and provided grounds for thinking absorption lines carried

information about the internal chemical structure of a substance. In his new lab, Hoppe developed tools for observing absorption lines in fluids (Fig. 2).

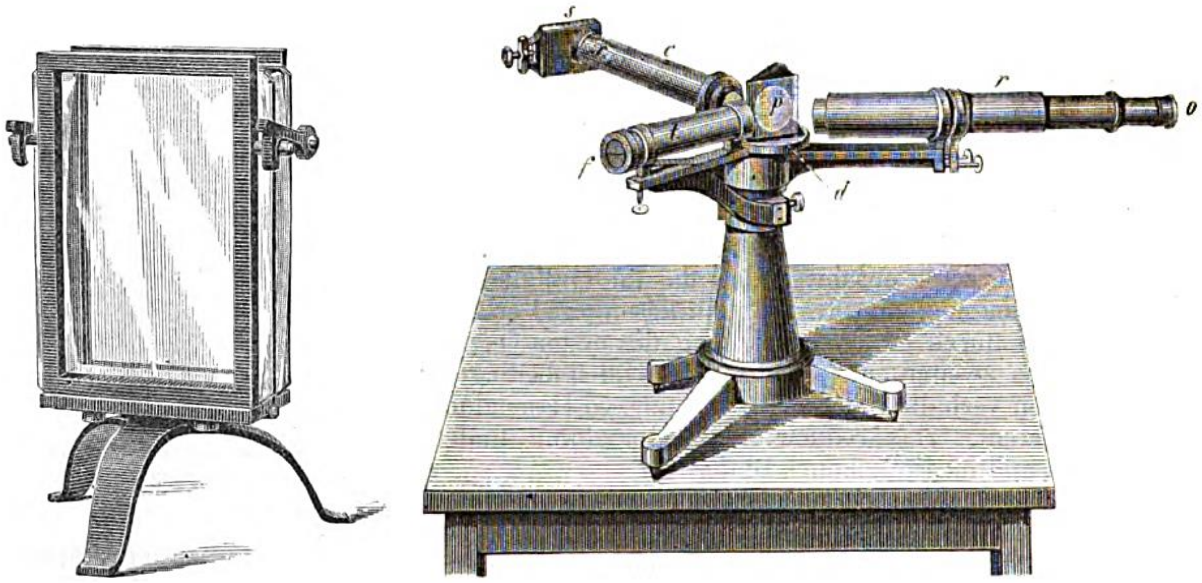


Figure 2: Left: the haematinometer, designed by Hoppe to hold fluid for spectral analysis. Right: A spectroscopic analyzer contemporaneous with Hoppe's work. Both from Funke (1876). Light enters through an adjustable slit (s or p on the left side of the drawing), passes through a layer of the sample, is prismatically refracted (at P), and viewed through an eyepiece (o on the right side). The center region of the device is covered during use.

Hoppe grew blood crystals, dissolved them in water, and recorded their absorption lines. They were the same as those of dissolved *Blutroth*. He then treated these preparations with various reagents; only those that broke down proteins caused the characteristic lines to disappear. Significantly, individual examination of the decomposition products of *Blutroth*—globulin and haematin—did not produce the same lines as dissolved crystals. For Hoppe, this indicated that the crystallization process was a method for obtaining purified samples of the protein *Blutroth*. The protein, he wrote, "is the same [substance] that forms Funke's crystals" (Hoppe, 1862, p. 449). He dubbed it 'haemoglobin' in 1864. The name and its identification with coloring matter was quickly accepted by leading researchers, as evidenced by Funke's textbook summary:

> The crystalline haemoglobin is itself the preformed coloring matter of the blood; It is only through chemical decomposition that it is broken down into a protein substance [globulin], which, depending on the nature of the decomposing agent, either remains in solution or coagulates, and an iron-containing pigment: haematin (Funke, 1876, p. 26).

The identification of blood crystals with *Blutroth* led Hoppe to refine his crystallization technique, checking his preparation with spectral data. He developed a method based on removing the fibrin from blood, separating the blood cells from the remaining serum with salt solution, adding an ether solution to dissolve the cell lining, filtering out *Blutroth*, and then putting it through multiple cycles of cooling in alcohol (to induce crystallization), filtration, dissolution, and recrystallization. In doing so, he gained confidence in the purity of the crystals. The dissolved crystals showed sharp spectral lines, consistent with his prior results, but now capable of revealing a difference between oxygenated arterial blood and venous blood (Holmes, 1995). This finding was reproduced by G. G. Stokes (1864), who concluded that the coloring matter of blood existed in two states, "distinguishable by a difference of colour and a fundamental difference in the action on the spectrum" (p. 357), and interchangeable via exposure to air or treatment with reducing agents. Researchers began referring to 'oxyhaemoglobin' and 'reduced haemoglobin' to distinguish these states.

3.3 Proliferation and stabilization of protocols

These episodes from the 1850s and 60s were instances in an overall proliferation of protocols for obtaining crystals. William Preyer's *The Blood Crystal* (1871), the first monograph dedicated to the subject, reviews six different crystallization methods. A later review by Arthur Gamgee (1898) mentions nine distinct procedures plus several modifications. Reichert and Brown (1909) refer to nineteen historical methods before introducing four of their own. Authors present a menagerie of seemingly scattershot techniques: scientists were freezing blood, cooling blood, heating, boiling, drying, coagulating, and electrocuting blood; adding alcohol, ether, salts, or bile; extracting and adding gases; drawing blood from live animals, from asphyxiated animals, from veins injected with water; crystallizing in a vacuum, under sunlight, under artificial light, in the dark, and other such variations of ambient conditions.

Authors reviewing these sundry forms of preparation often appear more interested in providing a catalog than methodological guidance. Reasons for preferring one protocol to another were sometimes unstated. Yet, from a historical standpoint, the variety of techniques present a dogged survey of blood

crystallization parameter space. In running through these procedures, researchers varied a wide range of conditions that could conceivably impact crystal growth—lighting, temperature, source animal, gaseous atmosphere, and so on.[14] Despite reticence from nineteenth century reviewers to declare a victor, some clear findings emerged: for example, early suggestions that light, oxygen, or the addition of carbonic acid aided crystal growth were discarded.[15] Other steps suggested in early texts, such as the removal of fibrin, became widely adopted. In a comparison of 11 procedures introduced after 1858, 7 explicitly refer to the use of defibrinated blood. Solutions had emerged for Lehmann's complaints about residual stroma contaminating crystals: by 1876, Funke had five ways to liberate the substance from cell membranes,[16] a combination of which became standardized as a process called "laking" the blood. 10 of the 11 reviewed procedures involve some way to separate crystal contents from cells. 9 of these involve some combination of freezing blood or mixing it with water, alcohol or ether solution—all methods suggested by Funke—and in all cases shortly after defibrination. Cooling, typically to a specified temperature of 0 degrees centigrade, likewise occurs in 7 of these procedures. Beneath the noise of diverse and novel techniques, commonalities emerged.

Emerging procedural stability is evidenced by the historical progression of blood crystallization protocols shown in Figure 3. The horizontal axis covers a series of protocols employed over time, from Hünefeld's discovery in 1840 to a joint paper by Kendrew and Perutz in 1948. The individual steps comprising each protocol have been stretched along the vertical axis, with the first step at the top, the last step at the bottom, and intermediates spaced evenly in between. Steps that are common to multiple protocols have been color-coded according to the type of operation—glass pressure, fibrin removal, drying, cooling, separation (by filters or centrifuge), and mixture with salts, water, alcohol, or ether.

[14] To this day biochemists make use of commercial arrays designed to expose samples to a systematically varying range of growth conditions.
[15] Rejection of these occur in Lehmann and Mitchell's writing, e.g.
[16] By then multiple authors, including Funke and Mitchell, had observed that the crystals could form inside red blood cells. This further supported the belief that haemoglobin originated from within the cells.

1840 Hunefeld	1851 Funke	1855 Lehmann	1858 Mitchell	1858 Mitchell2	1863 Funke	1864 Hoppe	1871 Rollet	1871 Bottcher	1871 Kuhne	1871 Anon	1886 Zinoffsky	1904 Salkowski	1909 R&B	1935 K&H	1942 Granick	1948 Kendrew
Place between glas	Place between glass	Chop clots	Place on slide	Place in open vesse	Dry cells	Defibrinate	Defibrinate	Inject water into an	Cool in ice	Defibrinate	Defibrinate	Mix with ether	Defibrinate	Defibrinate	Wash in saline	Wash in saline
														Wash in saline		
						Mix with salts					Mix with water + alcohol			Cool		
										Mix with water + alcohol		Cool to 0C		Mix with water + et	Dilute with water	Cool to -20C
		Wash		Expose to light and heat		Cool to 0C					Let cells sink			Centrifuge		
					Mix with water/alcohol/ether/salts		Mix with salts	Chloroform animal	Mix with salt solution	on	Decant	Mix with ether + alcohol		Extract fluid		
						Draw off supernatant				Cool to 0C	Add salts	Cool to 0C		Filter	Centrifuge	Filter
			Evaporate by half			Wash								Cool		
		Defibrinate		Let decompose		Add water + ether					Dilute with water			Add alcohol		
										Collect crystals	Heat to 35C	Filter		Cool	Discard supernata	Add pot. ferricy.
	Mix with water/alcohol/ether				Cool	Filter	Cool to 0C	Gather blood	Wash					Wait overnight		
											Add ammonia	Press out mother li	Mix with ether	Collect crystals		
						Cool to 0C				Dissolve in warm w	Mix with salt + sulf	Dissolve in water		Centrifuge	Dissolve in water	Dissolve in water
		Dilute with water		Evaporate										Wash in water + alcohol		
			Mix with water			Mix with alcohol					Cool to 0C			Mix with water		
										Cool to 0C		Filter		Heat to 39C	Centrifuge	Dialyze
					Evaporate	Cool to -5C to -10C	Thaw	Mix with water + al	Filter		Add alcohol			Add sodium hydroxide to dissolve		
		Expose to sunlight	and air	Remove drop							Wait 24 hours			Neutralize with hydrochloric acid		
						Collect crystals				Wait 24 hours	Wash with water +	Mix with alcohol		Centrifuge	Cool to 1C	Mix with salt
						Wash with water + alcohol								Cool		
											Repeat previous 7 steps			Repeat previous 4 steps		
Dry (in desiccator	Evaporate	Conduct carbonic a	Cover with glass	Evaporate	Add alcohol or salt	Dry	Repeat previous 2 s	Evaporate	Repeat previous 2	Collect crystals	Dry (in vacuum)	Cool to 0C	Centrifuge	Dialyze w/ water	Wait overnight	Evaporate

Figure 3: Select crystallization protocols from 1840 to 1948.

As seen in Figure 3, protocols generally lengthened and varied over time. While those dating to the 1840s and 50s could be summarized in at most six steps, protocols after the 1860s might involve thirteen or more. In time, protocols also began to show more commonalities with their predecessors, both in the types of operations that practitioners carried out and their relative ordering. Figure 4 modifies Figure 3 to better visualize these orderings. Each line follows the position of a type of operation within the protocols over time.

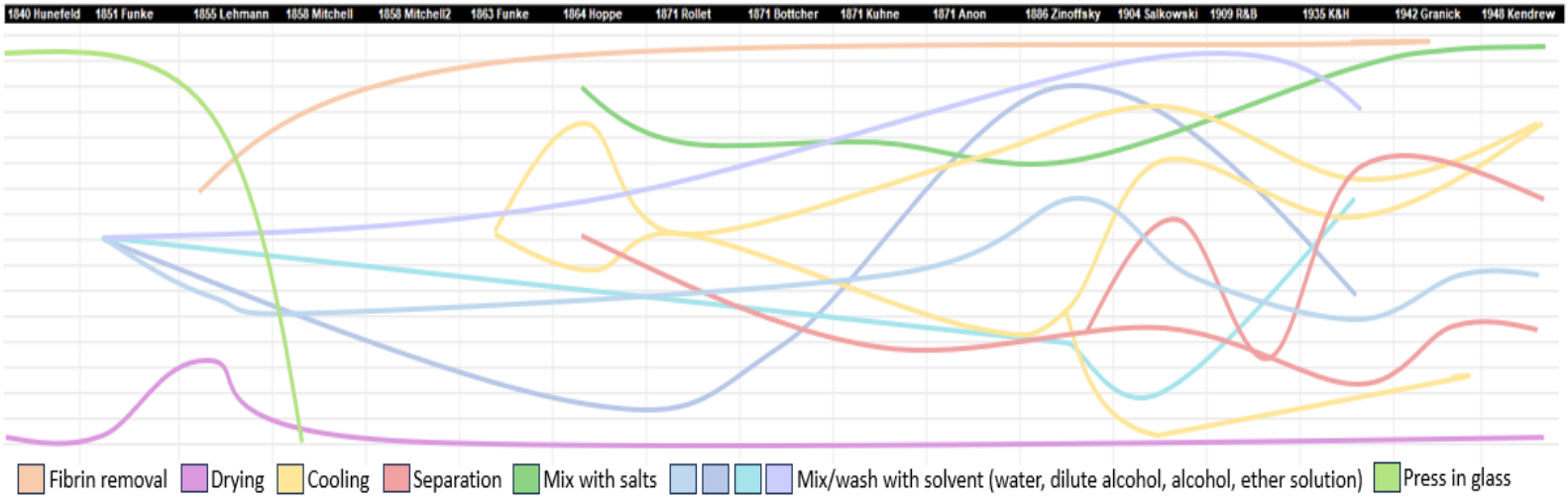


Figure 4: Trajectories of protocol operations, 1840-1948. Some repetitions of steps removed for clarity.

The extent that the relative position of a given operation persists over time provides a rough indication of procedural stability throughout this period. The less a colored line crosses over others, the more stable its

position across protocols. Some steps remained in the same relative position across most protocols in which they occur. Many in the middle of the protocols traded places repeatedly over time. This is a highly qualitative basis for historical inference, affected by the fidelity of handbooks. It is possible, for instance, that some steps were deemed obvious enough to go unlisted. Nevertheless, patterns of stabilization emerge within the inevitable gaps and variations.

For one, defibrination and evaporative drying (orange and purple, respectively) quickly assumed the positions of first and last step, with solvents (blue) used in between. New operations were layered into this framework. Following Hoppe in 1864, early application of salts became a common way to burst cell membranes after defibrination (indicated by the persistence of the dark green line toward the top of the order). Solvents (blue lines) often came immediately before or after salts (dark green) or were used as an alternative. Starting in the early 1860s, interleaved cycles of cooling, mixture with solvents, and filtration emerged as intermediary steps (indicated by the proximity of yellow, blue, and red lines). These steps became more densely layered over the following decades. A sequence of cooling, mixture with solvent, and then separation (yellow above blue above red) persists through the additions. Growth between glass plates (light green), on the other hand, was quickly jettisoned while other steps never gained traction.

More pronounced signs of procedural stability are found in a comparison of only Hoppe (1864) and Keilin and Hartree (1935) in Figure 5.

1864 Hoppe	1935 K&H
Defibrinate	Defibrinate
	Wash in saline
Mix with salts	Cool
	Mix water + ether
Cool to 0C	Centrifuge
	Extract fluid
Draw off supernatant	Filter
Wash	Cool
Add water + ether	Add alcohol
	Cool
Filter	Wait overnight
	Collect crystals
Cool to 0C	Centrifuge
	Wash in water + alc.
Mix with alcohol	Mix with water
	Heat to 39C
Cool to -5C to -10C	Add NaOH to dissolve
	Neutralize with HCl
Collect crystals	Centrifuge
Wash with water + al	Cool
	Repeat previous 4
Dry	Dialyze w/ water

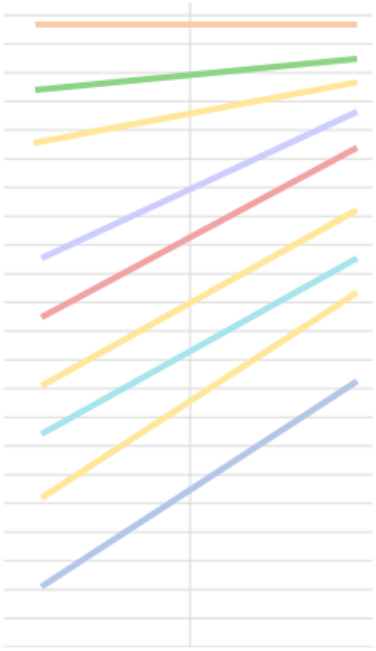


Figure 5: Comparison of protocols from Hoppe and Keilin & Hartree, showing the relative order of each of Hoppe's steps is preserved by K&H. The line chart does not show unshared operations.

This is evidence of continuity between protocols spanning seventy years. Ignoring the additional cooling and filtration/separation steps (aided by the new centrifuge), all nine of the steps common to Hoppe and K&H occur in the same relative order. Moreover, the authors selected here are distinctively influential within the history of protein crystallography. Hoppe's work identified blood crystals with haemoglobin. Three decades on, Salkowski's instructional *Practicum for Physiological and Pathological Chemistry* (1903), first published in 1893, cited Hoppe's as the standard method. Reichert and Brown (1909) devote a disproportionate amount of their historical overview of haemoglobin crystallography to an account of Hoppe's techniques, claiming them so successful that there had been little incentive to develop methods further. As for the others, David Keilin ran the Molteno Institute at Cambridge, which specialized in biochemistry. From 1938 to the early 1950s he provided lab facilities to Max Perutz, who eventually determined haemoglobin's structure. Perutz had not crystallized haemoglobin on his own before arriving in Cambridge, so it is likely Keilin had a direct influence his methods. There is reason, then, to believe the similarity between these protocols is no accident but rather shows a form of procedural stability running

through the tangle of crystallization steps that rose and fell between the mid-nineteenth and -twentieth centuries.

Procedural stabilization involves a tightening of parameter space—the range of causal conditions within which crystallization was carried out. How did it come about? Some of this was simply imitation of recognized success. The impact of Hoppe's work led to his technique being copied, with adjustments, in other significant results, as in Zinoffsky (1886), and presented as the main way to grow purified crystals (as in Salkowski's lab manual). But the restriction of protocols to a smaller range of operations and orderings was also justified by investigations of component steps. Physiologists would check the results of different operations under the microscope, developing a rationale for their actions that was lacking in prior works. Lehmann and Funke's microscopic observations that fibrin inhibited crystal growth justified the primacy of defibrination in subsequent protocols, including Hoppe's. Other operations were justified by reference to the chemical properties of blood contents. For example, it had been observed that less soluble blood (e.g., rodent blood, compared to human or cow) was generally easier to crystallize. As a result, authors began to recommend methods to reduce the solubility of the mother liquor to initiate crystallization. Prior procedures were reinterpreted accordingly. Thus, the "drying" or gradual evaporation of a droplet of blood in solution, employed from the start of crystallization, came to be viewed through the lens of solubility, as in Funke's textbook (1876).[17]

Researchers tracked and varied the conditions that gave rise to crystals and recorded the results. The study of these variances grounded causal generalizations over the discrete interventions comprising a protocol. Their impacts on crystal growth helped establish which of the conditions or manipulations played a prominent role in crystallization, and which could be consigned to background conditions with little effect.[18] For instance, Preyer grew crystals in a range of conditions, from total darkness to direct sunlight, in response to Lehmann and Mitchell's insistence on the role of light, saw little effect, and so relegated it to the background. Zinoffsky, in varying his methods, observed that washing cell contents

[17] This gave rise to techniques akin to present day "sitting drop" and "salting out" crystallization methods.
[18] Cf. Woodward (2000b) on the role of this distinction with respect to the kinds of causal generalizations that figure in experiment.

after laking was superfluous. Scientists' knowledge of causally relevant conditions is seen in those aspects of protocols that were strictly recommended (early defibrination was stressed for obtaining a pure sample), those where some flexibility was allowed (a range of temperatures below 0°C can be used for cooling; saline and ether solution are equally adequate for laking the blood), and those that fell into irrelevance (light and atmospheric conditions receive little attention after the 1850s). The cumulative effect was to restrict and control variance in conditions that had the most significant causal effect on crystallization outcomes, and to tolerate variance elsewhere, thereby establishing a particular causal niche in which satisfactory preparations were produced. Against a background of swarming and often irrelevant factors, there emerged a set of conditions that could be tuned to meet the desire for greater reliability—Lehmann's "exactness"—in two ways: greater consistency in the production of satisfactory crystals and in the outputs of procedures performed on these crystals.[19]

Through this self-scrutiny, protein crystallization stabilized into a relatively consistent set of procedures employed by researchers across the European continent and spanning the Atlantic. Blood crystals were consequently able to serve as stable objects of inquiry. We can summarize the process as follows: exploration of the parameter space of conditions accompanying a preparation procedure generates a set of causal generalizations. These state dependencies between empirically accessible factors such as ambient conditions, specific operations, and their effects observed in the resulting sample. Such generalizations justify altering steps within a protocol in a way that shifts preparation procedures toward consistent outcomes, or points of stability (here in the form of a reproducible object of sufficient purity). A body of local knowledge thus developed around crystallization protocols and at the same time aided in the characterization of blood crystal contents. First, in identifying factors affecting their formation, researchers acquired an initial understanding of what the crystals did and did not contain. It quickly

[19] The desire for reliability in Lehmann's sense recurs through this history. For example, Zinoffsky (1886) justified his methodological adjustments by citing inconsistencies in prior determinations of crystal iron and sulfur content. It is a testament to the claimed superiority of Zinoffsky's techniques that they yielded analyses of haemoglobin much closer to contemporary values. Where Preyer cites results finding 600 carbon atoms, 960 hydrogen, 154 oxygen, and 179 nitrogen in what is today called a single heme group, Zinoffsky measured 712C 1130H, 214O, and 245N. The contemporary values are 738C, 1166H, 203O, and 208N.

became clear, for instance, that they were distinct from blood fibrin or cell membranes. Second, as crystal preparation stabilized, they were more easily incorporated into further experimental procedures aiming to determine crystal properties and contents. They were studied with a growing set of techniques—the traditional analytical tools of organic chemists, the microgoniometer of the mineralogists, and novel methods of spectroscopic analysis. As crystal contents were further characterized, this fed back on preparation procedures, informing judgments over the purity of the crystals—their status as representatives of haemoglobin. Stability gave rise to new doubts, however. Persistent variations in data from multiple forms of intervention raised questions over the number of forms haemoglobin could take in its crystal morphology, chemical qualities, and its ultimate constitution or structure. Was haemoglobin one molecule, perhaps affected by various modifications and impurities, or many?

3.4 One haemoglobin or many?

Hoppe's work had established that blood crystals were preparations of haemoglobin; spectroscopic techniques distinguished two states of the protein, oxyhaemoglobin and reduced haemoglobin. With Stokes, this work had linked forms of haemoglobin to the physiological function of carrying oxygen in blood. But this was not the end of blood crystal varieties. From stabilized preparation protocols there emerged a multiplicity of microscopic, chemical, and spectroscopic details, and concomitant difficulties in explaining them.

Reactions to chemical tests and treatments only revealed qualitative knowledge about the nature of haemoglobin. Certain limits to insights afforded by elementary analysis were also becoming clear. In the 1830s, Liebig and Wöhler had established the phenomenon of isomerism: substances with the same formula could have different chemical properties. Determining haemoglobin's elements was not enough to explain its different characteristics. The main alternative to chemical intervention was crystallographic description. The compound microscope allowed researchers to observe wide varieties of minuscule crystals and depict these for readers (Fig. 6). Coupled with the goniometer (Fig. 1, right), this allowed for

a potentially deeper characterization that exploited Haüy's hypothesized link between crystal form and "molecular" structure.

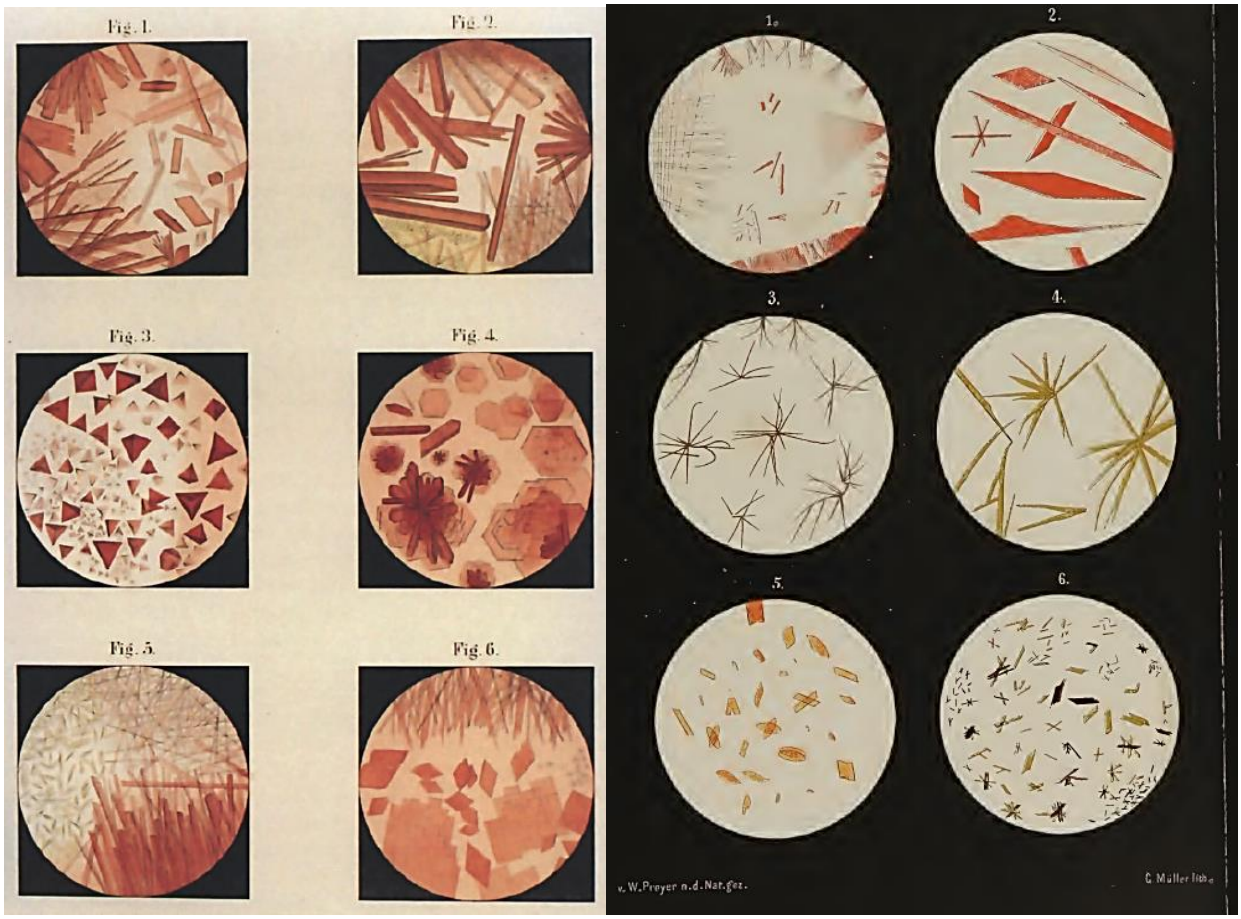


Figure 6: Left: drawings from Funke's Atlas of Phys. Chem. *(1853). Right: Preyer's* The Blood Crystal *(1871).*

The notion that chemical differences could be identified through crystal forms was a major inspiration to blood crystal researchers. Lehmann, after remarking that proteins may have the form of a complex with "several proximate constituents conjugated together," added,

> This view seems to gain support from the remarkable circumstance that there is an albuminous substance in the blood of carnivorous animals which crystallizes in prisms, while the corresponding substance in the blood of guinea-pigs and rats crystallizes in tetrahedra. This obviously points at combinations of an analogous kind, in which only one different constituent has entered, which, however, is the cause of the difference in the crystalline form of the otherwise perfectly analogous body (1855, p. 293).

In other words, Lehmann suspected that variation in crystal morphology was due to a small difference in the chemical constitution of an otherwise identical substance. If there was a link, as crystallographers

asserted, between chemical structure and crystal form, then the study of the different crystals found in animal blood could provide insight into the underlying chemical make-up of haemoglobin from diverse species.

This approach immediately raised questions. For one, what to make of the results from Silas Mitchell's friend, Johnston of Baltimore, for whom blood from two different sites in the same animal formed into different crystals? Many approached these questions with enthusiasm. Preyer wrote that studying blood crystals could pave the way to a new form of comparative evolutionary biology, which "sets variability alongside the constancy of proportions" within the temporal progression of animal forms:

> since *Blutroth* crystallizes in different systems in very closely related animals, and since their common ancestor had only one type of blood pigment, a material metamorphosis must have taken place at the same time as the change in form. And this means that, even if many simple connections between animal bodies are everywhere identical, the more relevant chemical constituents, especially the albumins and the coloring matter, are not merely different in kind, but are variable in time, variable in the same degree as the zoological species (1871, p. iv).

Like Lehmann, Preyer believed variation in crystal morphology was due to differences in the chemical constituents of animal haemoglobin. Going further, he posited that differences in crystal forms could provide evidence of subtle chemical evolutionary relationships underpinning similarities and differences in animal bodies.

Speculation on these latent chemical differences may have been emboldened by the rise of structural models in chemistry in the 1850s. These models settled into a regular graphical form with the popularization of Brown's structural notation in the 1860s: atoms represented by one or two letters, circled and connected by lines representing bonds (Ritter, 2001). The notion of isomerism was easily comprehensible in these terms: different arrangements of the same basic units could intuitively be seen to yield different kinds of molecules. But physiological chemists never arrived at detailed structural models of the molecules in blood crystals. Instead, studies carried on in the familiar classificatory format, supplemented with accounts of the different crystal shapes found in different species. Preyer's *The Blood Crystal* (1871) includes a table listing the crystals found in over forty animal species: their appearance, the fundamental crystal system they belong to, where they form, their solubility in water, and how easily

they crystallize. Despite ambitions for evolutionary insight, he could give no detailed conclusions about the underlying structural differences between blood of different animals nor how crystal features were connected to phylogeny. “There is little value to be placed on the information about the crystallizability of the haemoglobins of different animals because the same method was not always used […] the investigations carried out in relation to them usually suffer from so many errors that they prove nothing beyond what has long been known” (Preyer, 1871, p. 43). Future work, he hoped, would uncover whether the variations were due to distinct molecules or modifications of a molecule that was fundamentally the same.

Preyer’s book also includes an early quantitative elementary analyses of blood crystals in terms of a precise atomic weight. Where prior authors only gave proportional analyses, Preyer’s results—yielding the formula $C_{600}H_{960}N_{154}O_{179}FS_3$—suggested that haemoglobin was much larger than expected. Later authors carried out similar analyses on crystals from a range of species, encountering notable variation. By the end of the century, these methods were also plagued with uncertainty. Different authors’ analyses, the physiologist Gamgee complained, show “such extraordinary discrepancies in the results of ultimate organic analysis as to preclude a precise answer being given to such simple questions as the following: Is haemoglobin a body, having a constant composition in animals of the *same species*? Does the haemoglobin of *different animals* vary in chemical composition, and if so, within what limits?” (1898, p. 199). It was not clear whether these variations—differences in elementary analyses and crystal morphology within and across animal species—tracked genuine chemical differences or were artifacts of inexact methods. He attributed this to “differences in the purity of the substance analysed, and to errors of analysis” (p. 200) and argued that prior attempts at purification likely resulted in the analysis of a mixture of haemoglobin and products of decomposition. There was, then, deep uncertainty on the sources of the variation observed in crystal samples and their biological significance, which stoked continuing worries over purity.

3.5 Spectroscopic distinctions and refinements of protocol

Spectroscopy, by contrast, had enabled physiologists to infer some differences in the underlying chemical structure of blood crystals with greater confidence. Preyer devoted a large section of *The Blood Crystal* to an account of the spectral lines of haemoglobin, its products of decomposition, and modified forms resulting from treatments with salts, acids, and other chemicals. Hoppe's successor at Tübingen, Gustav von Hüfner, deepened this line of work, commissioning new spectrophotometric instruments, synthesizing novel forms of haemoglobin, and recording their distinct spectral properties. By the end of the nineteenth century, oxyhaemoglobin and its reduced form had been joined by methaemoglobin,[20] CO-haemoglobin,[21] NO-haemoglobin, CO-methaemoglobin, and NO-methaemoglobin.

Spectroscopy opened new inquiries into crystal preparation techniques. A simple example is found in Preyer's spectral line illustrations (Fig. 7). Lines 2-8 correspond to gradual increases in the concentration of dissolved oxyhaemoglobin crystals in clear solution. These show a widening of absorption bands as the effect, up to a point where the outcome resembles the large gap seen in the *reduced* haemoglobin spectrum in Line 9. This showed how an overly dilute or overly concentrated sample could yield errors in spectroscopic inference.

[20] Studied by Hoppe in 1866.
[21] Studied, but without being aware of it as such, by Hoppe in the late 1850s.

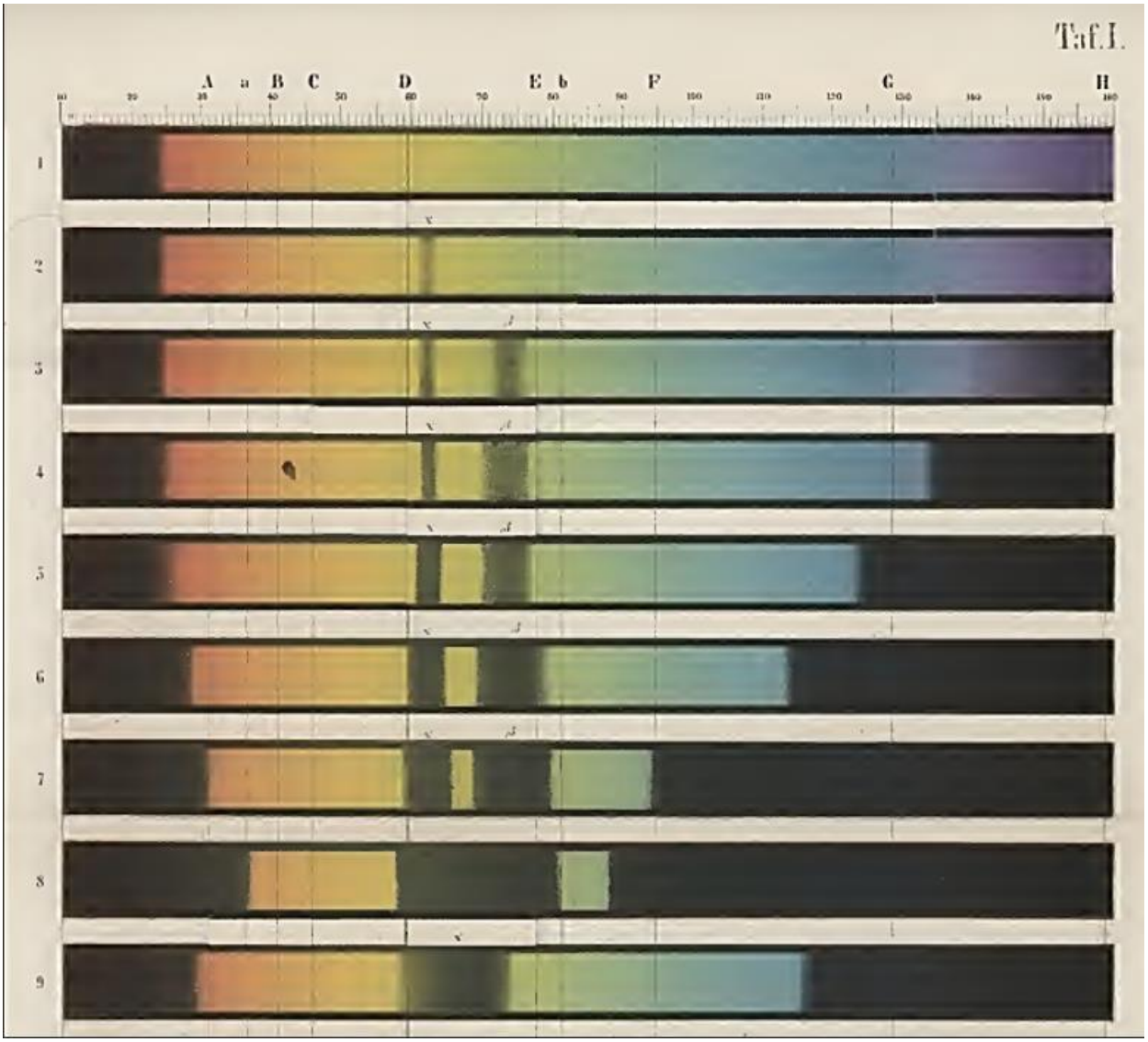


Figure 7: Spectral lines from Preyer (1871) showing increase in the concentration of oxyhaemoglobin in solution and comparison to reduced haemoglobin.

Von Hüfner and others applied quantitative methods to spectroscopy, introducing new ways to check the purity of a crystallization result. This included the derivation of quantities like the extinction coefficient, found to be characteristically constant for definite concentrations of a given substance. Alarmingly, spectral data showed that some previously accepted preparation procedures were likely altering crystal purity:

> Since spectrophotometry has supplied us with a method of determining, with an accuracy previously unattainable, the purity of a colouring matter, it has been found that although oxyhaemoglobin which has been recrystallised, when examined in the ordinary manner, exhibits a spectrum which appears identical with that of the colouring matter which has

> been only once crystallised, its spectrophotometric constants have changed; in other words, when oxyhaemoglobin is recrystallised it undergoes a change, possibly only affecting its physical, but more probably affecting its chemical constitution also (Gamgee, 1898, p. 197).

Following Hoppe, many recommended cycles of dissolution and recrystallization to fully separate haemoglobin from other components of blood. Zinoffsky (1886), motivated by perceived impurities in prior methods, preparation and analyses, repeatedly recrystallized his samples prior to analysis. Spectrophotometry called this into question and likely hastened broader adoption of the centrifuge as an alternative method of separation.

Evaluations of the crystal's purity, their ability to stand for the target of inquiry, evolved with the examination of old methods by new techniques, inducing further alterations to preparation procedures. Spectroscopic analysis provided a new way to characterize differences in haemoglobin structure by means of absorption bands *and* provided evidence that certain methods of preparation, like Zinoffsky's, altered haemoglobin structure in ways that were previously undetectable. These findings thus fed back into researchers' understanding of the preparation process, revealing new sources of variation to control. Again, preparation and characterization could advance in a reflexive loop, where novel forms of evidence of haemoglobin structure carried direct implications for the procedures used to prepare it. Knowledge of the object of study was still bound up with knowledge of the techniques for preparing it. On the other hand, as preparation procedures were further refined and stabilized, it became increasingly apparent that some sources of variation could not be reduced to impurities or error.

3.6 A culmination of the nineteenth century approach

Lehmann (1855) had assumed a link between crystal form and chemical constitution. Fifty years later, this assumption remained operative. In 1902, the American biochemist Thomas Osborne wrote of edestin that "the form of the crystal is determined by the protein molecule" (1902, p. 55). The same idea motivated Edward Reichert and Amos Brown's (R&B) sprawling study of blood crystals in 1909: "Differences of chemical constitution are accompanied by differences of physical structure, and the crystallographic test

of differences of chemical constitution is recognized as the most delicate test of such differences" (1909, pp. 144-145). Why is this? Because "*Substances that show differences in crystal structure are different chemical substances*" (p. 145).

As a post-graduate, Reichert had studied in Leipzig and Berlin (Frank, 1987) and subsequently received training from Henry Newell Martin, first professor of physiology at Hopkins, himself a student of Michael Foster of Cambridge. Like his German forebears, Reichert had a taste for exhaustive applications of method. R&B's *Crystallography of Hemoglobins* (1909) thoroughly reviewed prior works on the subject before detailing the blood crystals from several hundred species, many obtained from the Philadelphia zoo. The work was supplemented with over 100 plates of photomicrographs. This was the first of two volumes in which Reichert sought taxonomic insight from chemical differences, an aim shared by Preyer and contemporaries such as George Nuttall (1904).[22]

R&B took great care to ensure they were working with pure samples. By adding and subtracting steps to their procedures and comparing the results, they were able to winnow down the crystal preparation process. Where prior methods regularly advised the use of alcohol or alkali salts to initiate crystallization, R&B avoided them due to deleterious effects. Alcohol, for instance, lowered the extinction coefficient, decolorized the crystals over time, and affected the water that sometimes crystallizes with haemoglobin. When they couldn't defibrinate blood, they made use of anti-coagulants like oxalate—but not before inspecting its effects on crystals and determining that its only impact was on the proportion of oxyhemoblin in horse and mule blood. They worked quickly to circumvent the deterioration of crystals over time, and avoided recrystallization at all costs, opting for the centrifuge instead. In short, they built on and scrutinized prior procedures, in the process producing the most extensive survey of blood crystals to date.

Working with so many animals gave R&B the ability to make certain methodological generalizations:

[22] The second Reichert work in this vein was a study of plant starches from 1919.

> We found, as we gained experience with the bloods of different species, that, while the blood of each species must be treated as an individual, we could nevertheless depend with some confidence upon the guidance of certain generalizations in the selection of the best method to be pursued (1909, p. 141).

For example, they mention finding that their methods of crystallization were effective for some orders of species (such as Rodentia, Canidae, and Felidae), while difficult to apply to the entire class of birds. They also found the best data was produced from crystals formed at room temperature, but that observations ought to be made in near freezing temperatures. Despite these advances, the work is an outgrowth of nineteenth century research on blood crystals, and the authors encountered familiar constraints in drawing their conclusions.

R&B used a specialized crystallographic device that combined numerous functions, "a form of microscope which is at once a goniometer, a polariscope, and an instrument for measuring optic axial angles—in short for determining the physical crystallographic constants of small crystals" (1909, p. 145). After haemoglobin crystals were prepared, these were put under the microscope to determine their plane angles, the relations of the parts of composite crystals to one another, their pleochroism (i.e., change in color under polarized light), the positions and relative values of their light elasticity axes (a refractive property), and the position and angle of inclination of their optical axes. Handling so many blood crystals allowed R&B to correct inconsistencies in prior observations. Claims that the crystals could be colorless, for example, were explained in terms of pleochroism and the relatively faint color of metoxyhaemoglobin.

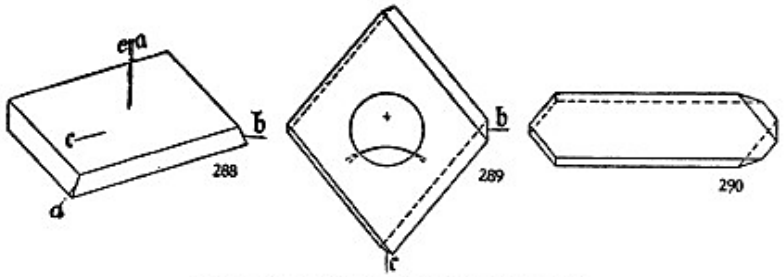
Two sample pages give a sense of the content of their study (Fig. 8; Fig. 9). For each species, crystallographic features were described in detail, accompanied by illustrations and photomicrographs. Within each section, summary tables allowed for interspecies comparisons.

Oxyhemoglobin of Ursus maritimus.

Monoclinic hemimorphic (monoclinic sphenoidal): Axial ratio $a : b : \dot{c} = 1.2088 : 1 : \dot{c}$; $\beta = 73° 2'$ (calculated).

Forms observed: Unit pyramid (T11), imperfectly developed; unit prism (1T0), prism (230), in twins only; basal pinacoid (001); also rarely (100) ? or (101).

Angles: Traces of the unit prism on the base, edges 110–001 ∧ T10–001 = 79° 12½', average of a number of measurements; 110 ∧ 001 = 77° (about); T11 ∧ 001 = 48° 30'. The angle of the dome (101), or orthopinacoid (100), on the base was not determined.



FIGS. 288, 289, 290. *Ursus maritimus* Oxyhemoglobin.

Habit tabular on the base, the crystal consisting of the basal pinacoid (001) cut by the unit prism at one end of the ortho-axis and by the unit pyramid at the other end (text figures 288 and 289). The first crystals to form are of this habit, porous-looking tabular crystals that are proportionately rather thick, but they do not attain very large size. They are usually in trillings as already described for the black bear oxyhemoglobin (text figures 286 and 287) and this kind of twinning is normal in the bears. As these crystals of the first crop disappear, they are succeeded by crystals of the second crop, in which the base (001) and the unit prism (110) are well formed, and usually have sharp angles and smooth faces; but the unit-pyramid planes are generally imperfect in these second-crop crystals. Nevertheless, the angle of the unit pyramid on the base was obtained from crystals of the second crop. The tabular crystals of the first crop are proportionately much thicker than those of the second crop, the ratio of the long diagonal of the plate to its thickness being about 5 : 1; while in the second-crop crystals this ratio will average nearer to 25 : 1. The crystals of the second crop produce parallel growths, but do not twin, as do those of the first crop. A few crystals were observed in the second crop that were prismatic on the ortho-axis, by development of planes in the zone 100–001, which seemed to be (100) or (101), and (001) (text figure 290); but the angle on the base was not measurable, owing to the position in which the crystals were lying.

Pleochroism was rather marked in positions where the axis of greatest elasticity appeared in the section; the colors were: a nearly colorless, b and c about equal and deep red, somewhat brownish-red. The crystals of the first crop, which look soft and porous, do not show as strong double refraction as those of the second crop; the extinction on the flat is symmetrical with the outline of the plate; on edge views of the plate the extinction runs up to 20° with the axis *a*, or the long dimension of the plate. The orientation of the elasticity axes is $c \wedge a = 20°$, in the obtuse angle; $b = b$, $a \wedge \dot{c} = 0° 6'$ (calculated); or a is parallel to $\dot{c}$ as nearly as can be measured. On the flat aspect, in convergent light,

Figure 8: Properties of black bear blood crystals (Reichert & Brown, 1909).

TABLE 42.—*Crystallographic characters of the hemoglobins of the Rodentia.*

Name of species.	Axial ratio $a:b:\dot{c}$, etc.	Prism angle or traces of prism on base (normals).	Angle β.	Extinction angle.	Optical character.	System and class.	Substance
		° ′	°				
Sciurus vulgaris	.....	60 0	90	0°	Weakly positive	Hexagonal or pseudohexagonal	α-OHb.
Do	0.577 : 1 : $\dot{c}$	60 0	90	0°	Negative	Orthorhombic	β-OHb.
Sciurus rufiventer neglectus	.....	60 0	90	0°	Weakly positive	Hexagonal	OHb.
Sciurus carolinensis	0.577 : 1 : $\dot{c}$	60 0	90	0°	Negative	Orthorhombic	OHb.
Sciuropterus volans	.....	60 0	90	0°	Do.	Hexagonal	OHb.
Tamias striatus	0.9246 : 1 : 0.589	85 30	90	0°	Positive?	Orthorhombic	OHb.
Cynomys ludovicianus	.....	... 0	90	0°	.....	Do.	OHb.
Marmota monax	.....	60 0	90	0°	Negative	Hexagonal or pseudohexagonal	α-OHb.
Do	.....	... 0	..	0°	Positive	Orthorhombic	β-OHb.
Do	1.804 : 1 : $\dot{c}$	122 0 (58* 0)	near 90	$c \wedge a = 11°$	Negative	Monoclinic	γ-OHb.
Castor canadensis	1.732 : 1 : $\dot{c}$	120 0 (60* 0)	78	$a \wedge a = 8°$	Positive	Do.	OHb.
Fiber zibethicus	1.6318 : 1 : $\dot{c}$	117 0 (63* 0)	68	$c \wedge a = 15°$	Negative	Do.	OHb.
Mus norvegicus albino	0.7829 : 1 : 0.7332	76 7	90	0°	Positive	Orthorhombic	OHb.
Mus norvegicus	0.7829 : 1 : 0.7332	76 7	90	0°	Do.	Do.	α-OHb.
Do	1 : 1 : 1	90 0	90	..	Isotropic	Isometric	β-OHb.
Mus rattus	0.7829 : 1 : 0.5864	76 7	90	0°	Positive	Orthorhombic	OHb.
Mus alexandrinus	0.7829 : 1 : 0.5880	76 7	90	0°	Do.	Do.	OHb.
Erethizon dorsatus	0.5543 : 1 : $\dot{c}$	58 0	56	$b \wedge a = 20°$	Do.	Monoclinic	α-OHb.
Do	0.8170 : 1 : $\dot{c}$	78 30	90	0°	Do.	Orthorhombic	β-OHb.
Cavia cutleri, domesticated variety	.....	.....	90	0°	Do.	Do.	OHb.
Hydrochœrus capyvara	1 : 1.8184	90 0	90	0°	Negative	Tetragonal	α-OHb.
Do	.....	.....	90	0°	Do.	Orthorhombic	β-OHb.
Lepus cuniculus	0.643 : 1 : 0.797	65 30	85	$a \wedge \dot{c} = 15°$	Positive	Monoclinic	α-OHb.
Do	0.5317 : 1 : $\dot{c}$	56 0	90	0°	Do.	Orthorhombic	β-OHb.
Lepus europæus	0.6588 : 1 : 0.8069	66 0	85	$a \wedge \dot{c} = 15°$	Negative?	Monoclinic	α-OHb.

*True angle of traces of prism on base.

Figure 9: Comparative table showing crystallographic characteristics of rodent haemoglobin (Reichert & Brown, 1909).

R&B's strategy for inferring the chemical constitution of haemoglobin from crystal form was based on the principle cited earlier, that "*Substances that show differences in crystal structure are different chemical substances*" (1909, p. 145). The major claims asserted in their study on this basis are, first, that crystal morphology varied with different batches of blood collected from the same species. As with prior researchers, R&B inferred a change in the constitution of the haemoglobin:

> It was found in the case of many species that the fresh blood would first crystallize in one form of oxyhaemoglobin; that later a second crop of crystals would appear having a totally different habit and even crystal system, or, in other words, different constitution; and that sometimes this would be succeeded by a third crop having a still different form (p. 323).

Second, they claimed that blood from a given genus crystallized into a common form, but not without variation between species:

> The crystals obtained from different species of a genus are characteristic of that species, but differ from those of other species of the genus in angles or axial ratio, in optical characters, and especially in those characters comprised under the general term of crystal habit, so that one species can usually be distinguished from another by its haemoglobin crystals. But these differences are not such as to preclude the crystals from all species of a genus being placed in an isomorphous series (p. 327).

Third, they took their results to indicate that haemoglobin molecules had variable structure, though they were unable to say what these variations consisted in:

> In large molecules like those of the haemoglobins, *plasticity of the molecule* is very likely; moreover, there is no doubt from the recorded observations of the practical *plasticity of the crystal structure* […] Their elementary composition may be various or they may be stereoisomers of the same centesimal composition, but all are connected by the common nucleus hemin, whose crystals show angles that belong in the same isomorphous series (p. 332).

R&B had given the most expansive account to date of the varieties of animal blood crystals. Their crystallographic work gave grounds for thinking, with Preyer, that interspecies variation in crystal form might correspond to phylogenetically significant forms of chemical differentiation. However, they could do little to explain the perplexing variations in crystal morphology. Their inferences from crystallographic to constitutional differences were compromised by a fundamental vagueness around the notion of "constitution." R&B do not offer insight into how features corresponding to "constitution" or being "different chemical substances" could account for the observed differences between haemoglobin crystals, much less clarify phylogenetic relations among animals. Despite the assumed link between crystal properties and chemical structure, their compendium offered little insight into the chemical constitution of haemoglobin and relationships between animal species. By the middle of the twentieth century leading haemoglobin researchers had abandoned this guiding assumption.[23]

As more varieties of crystal morphologies and haemoglobin types were uncovered, they became fodder for burgeoning inquiries into chemical structure and animal phylogeny. Data from nineteenth century crystallography raised questions about the chemical nature of haemoglobin but offered limited

[23] Kendrew and Perutz (1948) routinely cite R&B in characterizing their haemoglobin crystals but cast doubt on this assumption. They note it sits uneasily with large morphological variation in haemoglobin crystals derived from the same fetus. By 1952, this assumption was abandoned by Cambridge crystallographers, who framed the notion as counterintuitive: "[diffraction pattern] similarities support the conclusion that haemoglobins from different species of mammal are very similar in general structure, as might have been expected" (Bragg & Perutz, 1952, p. 426).

insight. Haüy speculated about the "structure" of crystals constituents in the late 18th century. Subsequent crystallographers gestured at the notion of protein structure or "internal constitution," but acknowledged there was no crystallographic data to answer their questions at sufficient grain. Instead, researchers were confined to examining optical and morphological features of crystals themselves, forming classifications therefrom. R&B's exhaustive inquiry yielded muted conclusions on the relation between chemical structure, animal taxonomy, and crystal variations.

Some retrospective reasons for this might be offered: Given the facts of polymorphism and isomorphism in crystals, morphology was unreliable for understanding chemical structure. Similarly, the structural complexities of proteins were not recognized—much less inferable from crystal forms—by a community skeptical of the possibility of macromolecules. Other reasons involve research context: Blood crystals were strange objects, sitting at the boundary between the lifeless matter of mineralogy and the "animal substances" of physiological chemists. Reichert was a physiologist by training and Brown a mineralogist. Their collaboration reflected the dominant modes of inquiry into blood crystals in the nineteenth century. They were also aging in 1909; by 1920 both had exited academia. Meanwhile, biochemistry emerged, in which crystallographic work was less celebrated than in the physiological tradition. This may have been due to the extensive labor it required. When researchers turned to novel substances, they were confronted anew with uncertainties from the early days of blood crystallization. Despite a surge in animal protein identifications, only three noteworthy cases had been successfully crystallized by 1899 (Fruton, 1999). There were conceptual obstacles as well: the scholarly community was riven over whether proteins were ultimately of the same nature as the systems studied by inorganic chemists. Such views gave way to a conception of living proteins as amorphous protoplasmic colloids, fundamentally different in their chemical behavior from "crystalloid" matter.[24] It was doubted that

[24] This view's influence on R&B is evident, despite their focus. They describe cellular protoplasm as "consisting essentially of an extremely complex solution of interacting and interdependent colloids and crystalloids" (Reichert & Brown, 1909, p. iii) and claimed haemoglobin is "amorphous in the corpuscles, or is 'colloidal,'" under certain conditions (p. 338), colloids being "the typical condition of living matter" (p. 337).

important biological questions could be answered through the study of crystalline samples and crystallization fell out of favor for a generation.

4 Technical knowledge, characterization, and explanatory pursuits

I have used this case study to support two main claims about the epistemic role of preparations, which I will now consider at a higher level of generality. The first claim is that attention to preparations demonstrates how technical knowledge can be tightly connected to knowledge of a scientific object or phenomenon such that scientists improve their understanding of what they are preparing by better understanding their preparation procedures. How should we describe the knowledge involved in turning blood into samples of crystalline haemoglobin? Recent work by Jutta Shickore (2007; 2017) has highlighted an important step in scientists' learning about the world: grappling with experimental materials and instruments to determine "the status, merits, possibilities and limits of their investigative tools" (2007, p. 3). Reflexive efforts, responsive to what she terms "second-order concerns" about methods, yield a form of technical and procedural knowledge that ultimately "finds its expression in instruction manuals" (2007, p. 13). The history of blood crystallization illustrates a dynamic between scientists' second-order understanding of preparation techniques and their characterizations of a specific object of inquiry.

In the case of preparations, we see these forms of knowledge—of techniques and object—bound together in a mutually reinforcing pattern. As a successful preparation practice develops, it coheres into a sequence of actions guided by a growing set of causal generalizations. These generalizations describe stable regularities between components of an empirical setup, including the instruments with which, objects to which, and environmental conditions in which the sequence of actions takes place. Researchers learn "a large number of highly specific local empirical facts about the causal characteristics" of the process (Woodward, 2000a, p. S170). As these generalizations accumulate the distinction between foreground and background conditions sharpens, an object or phenomenon of inquiry comes to assume an increasingly defined role within the causal nexus under description.

Such causal claims initially concerned the conditions within the crystallization parameter space. Scientists did not know *what* they had separated upon discovering crystals in dried blood. The quality of crystals was assessed in terms of visibility features under the microscope, like form and color. Traits like redness were tightly associated with crystal contents, loss of which indicated impurity. As researchers converged on protocols that produced samples with these prescribed traits, blood crystals became a relatively stable node in a broader network of data-generating procedures. This allowed for reliable use of the crystals as objects of inquiry, securing inferences from data to claims about their contents. Crystallization protocols joined with chemical, microscopic, and spectroscopic analyses, enabling further parsing of the components of blood and the identification of distinct types of haemoglobin. These investigations served multiple purposes. First, they expanded the range of empirical results used to characterize the properties and contents of blood crystals. For example, Hoppe's spectroscopic analysis of blood crystals supported the claim that the crystals contain the coloring matter of blood, since they produced the same spectral data as alternative preparations of *Blutroth*. Second, they contributed to knowledge of preparation procedures. Downstream intervention on prepared samples, coupled with an understanding of relevant causal factors involved in their preparation, allowed researchers to identify further sources of impurity and morphological artifacts.

Because a preparation is known through this extended causal role, changes in scientists' understanding of techniques can alter their understanding of the object of inquiry; insights into this object from one technique may reveal errors in another; recognized limitations of techniques act as empirical constraints on scientists' characterization of a target phenomenon. Here the prepared sample has both causal and inferential functions: target of interventions and presumed source of data, a "black box" that becomes gradually characterized by exploring its causal role and inferring features therefrom. In these ways, establishing the representative status of a preparation—characterizing it as sample *of* something—is bound up with knowledge of preparation techniques. Prepared samples, such as blood crystals, blend

these forms of knowledge insofar as they both stand for an object of inquiry and embody the technical knowledge required to faithfully prepare it.[25]

The second claim is that attention to preparations helps to demonstrate the ways that characterization is a relatively autonomous domain of scientific activity. Diverse lines of data production branched off from prepared blood crystals and were compared. These had two effects: first, they expanded and layered the set of characteristics associated with purified crystal contents. Through comparison with chemical and spectrographic analyses, the crystals were identified with a protein component of blood: "*Blutroth*" or "coloring matter." Rebaptized as haemoglobin, it became associated with empirical characteristics—a specific chemical constitution, spectrographic signature, and so on. Using these results, researchers judged the purity of their samples in terms that went beyond superficial traits like color, and revised preparation procedures accordingly. Second, as crystals were incorporated into novel techniques and their empirical characteristics were refined, this generated questions about various sub-types of haemoglobin. Some differences in characteristics were not interpreted as impurities, but rather as different species of crystalline haemoglobin, varying with source animal and preparation treatments. Spurred by the classificatory ambitions of nineteenth century mineralogy and chemistry, questions about the empirical qualities distinguishing, say, oxyhaemoglobin crystals from reduced haemoglobin crystals in rodent blood became salient.

This shows how the activities and knowledge involved in characterizing an object have autonomy from other scientific practices. Preparations can function as a site for integrating several different forms of experiment. When blood crystals were incorporated into a broader array of techniques, this informed the process of their preparation and the conception of their contents, but it didn't tie them to any one form of data collection; if some methods fell out of favor, knowledge of blood crystallization could be transferred to a new experimental paradigm largely intact. Further, the production and use of these preparations illustrates the independence of characterization from explanatory pursuits. At times, characterization was pursued for its own sake; researchers simply wanted to know *what* they were crystallizing. In the process,

[25] Cf. Baird (2004).

they generated data that called for novel explanations (for instance, the physiological role of haemoglobin versus methaemoglobin). Efforts to characterize blood crystals thus made a positive contribution to explanatory pursuits, expanding the range of questions under consideration. Yet, because this work was often entwined with specific technical knowledge, it also constrained the questions that could be fruitfully pursued.

The multiple forms of data generated from blood crystals raised deeper explanatory questions: what was the relation between crystal morphology and chemical constitution? Could the crystallographic variations in animal blood be explained in terms of differences in the molecular structure of haemoglobin? Was the haemoglobin forming these different crystals the same fundamental molecule with some modifying component, or was it a different molecular structure in each case? And could these differences provide a chemical explanation for phylogenetic relationships between animals? Yet the techniques used to characterize blood crystals imposed a set of constraints on the questions that could be fruitfully pursued. Evidence in the form of crystallographic properties, spectroscopic constants, and elementary analyses grossly underdetermined these structural questions. The classificatory fervor of R&B's crystallography had little impact on conceptions of haemoglobin structure or animal phylogeny; their guiding assumption linking crystal structure to chemical structure was later abandoned.

It is tempting to conclude that this research program ended in failure, but this would be too clean a retelling, one that privileges the theoretical explanations over all else. These researchers had accumulated a detailed body of knowledge on the production and manipulation of a notoriously difficult object—crystallized animal protein. In the latter 1920s a new generation of scientists took interest in these samples. The theoretical and explanatory context had shifted, new instrumentation had come to the fore, yet the techniques for growing crystals were noticeably familiar, as seen in the comparison of Hoppe's protocol to those of twentieth century biochemists and X-ray crystallographers (Section 3.3). Blood crystal preparations and the empirical characterization of their contents persisted as an autonomous body of knowledge across these shifts in explanatory and data gathering pursuits.

5 Conclusion

In sum, the discourse and practices of sample preparation are longstanding features of science, yet these remain relatively unexplored by historians and philosophers. Two noteworthy features of the epistemic role of preparations have been highlighted here. First, preparations exemplify a mutually reinforcing relationship between procedural or technical forms of understanding and the characterization of an object of study. Especially in the early stages of a developing preparation, scientists' knowledge of what is prepared is inseparable from their technical knowledge. As a preparation procedure matures and stabilizes, the prepared sample takes on this dual character as the representative of a natural object and the embodiment of this technical knowledge. Second, stability in preparation outputs contribute to preparation's autonomy from other forms of scientific activity. Prepared samples may be amenable to multiple forms of data collection, each of which might characterize the sample in different ways. These characterizations may spur or constrain different explanatory pursuits, though they are often pursued and preserved without reference to these. This dovetails with and contributes to recent work in the epistemology of science that emphasizes the role of technical knowledge in basic activities like characterization. Given their long history and contemporary role as nodes in the multi-instrumental integrative practices of the life sciences, preparations deserve further attention.

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