2 Early Scientific Investigations

2.1 Advances in the 18th Century

Following *Paracelsus*, the early investigators of medicinal chemistry were often largely trained as physicians who had gained a knowledge of the emerging field of chemistry, a field yet in its infancy. By the middle of the last century of the Enlightenment (*Zeitalter der Aufklärung*), an area of critical questioning and an age of clearing up, chemistry was a field in revolution, poised for rapid development from its 16th and 17th century alchemistry antecedents and stimulated by a cascade of discoveries from many notables, including the seminal contributions of the French nobleman scientist *Lavoisier*[1] and his findings, *inter alia*, on stoichiometry, the law of conservation of mass, respiration and on gases (*24*, *27*), and the discovery of oxygen (*27*) by *Scheele* (*Carl Wilhelm Scheele*, 1742–1786) in Sweden and the English natural philosopher *Priestly* (*Joseph Priestley*, 1733–1804) in England. Although it could not have been recognized at the time, *Lavoisier*'s studies of combustion became the basis for what was to become perhaps the most important quantitative method for analyzing carbon compounds in the 19th and early 20th centuries and the first quantitative analytic technique applied to bile pigments: named elemental combustion analysis. *Lavoisier* was the first to explain combustion as a process of combination with oxygen, that led to the abandonment of the long-held phlogiston theory, which like many fiercely-held beliefs died only slowly. Successfully applying his knowledge of the combustion process, *Lavoisier* devised an apparatus in which *weighed quantities* of natural products such as spirit of wine, oils, fats, sugars, *etc*. were combusted in air and the CO_2 and H_2O products formed were *weighed*. From the *weights* involved, the %C and %H of the original substance could be calculated, and from the atomic weights of carbon, hydrogen, and oxygen, an empirical formula for the substance could be derived. The basic

[1] *Antoine-Laurent de Lavoisier*, the founder of scientific chemistry, was born on August 26, 1743 in Paris and died on May 8, 1794 in Paris. He studied chemistry, botany, mathematics, and astronomy at the Collège de Mazarin from 1754 to 1761, was elected to l'Académie Française des Sciences at age 25 and commenced his famous investigations on combustion at age 30. He was a strong advocate of quantitative (weighing and measuring) methods and of experimental work.

D.A. Lightner, *Bilirubin: Jekyll and Hyde Pigment of Life*, Progress in the Chemistry of Organic Natural Products, Vol. 98, DOI 10.1007/978-3-7091-1637-1_2,

principle and seminal experiment became the basis for the modern process of combustion analysis introduced some 50 years later by *Liebig*.[2]

Lavoisier's genius and adherence to experimentation were revealed in his influential *Traité Élémentaire de Chimie* in 1789 (*24*), the first modern chemistry textbook that so clearly and logically set forth principles which were fully confirmed in later times. "Il n'est jamais permis, en physique et en chimie de *supposer* ce qu'on plut *déterminer* par des experiences directes." [It is never allowed in physics and in chemistry, to *suppose* what can be *determined* by direct experiment.] France lost its most prominent scientist during its revolution. Denounced by his colleagues during the "Reign of Terror" (including *Antoine François le Comte de Fourcroy*, who was among the first to separate the components of bile and gallstones, *see below*), *Lavoisier* at age 50 was tried, convicted, and sent to the guillotine on May 8, 1794, some 16 years to the month after the death of *Voltaire* (*François-Marie Arouet*, 1694–1778), one of the most prominent individuals of the French Enlightenment and a forerunner of the French Revolution of 1789–1799. The famous Italian-French mathematician *Lagrange*[3] wrote: "Cela leur à pris seulement un instant pour lui couper la tête, mai la France pourrait pas en produire une autre pareille en un siècle." [It took them only an instant to cut off his head, but France will probably not produce another like it in a century].

Such was the importance of *Lavoisier*'s contributions that *Kekulé*[4] described him in his famous textbook (*28*) ". . . *Lavoisier*, der eigentliche Begründer wissen-

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[2] *Justus von Liebig* was born on May 12, 1803 in Darmstadt and died on April 18, 1873 in Munich. He studied under *Kastner* from 1819 to 1822 at the universities in Bonn and in Erlangen, where he received the doctorate, engaged in advanced chemistry studies in *Gay-Lussac*'s laboratory in 1822, became a.o. Professor at Giessen (since the end of World War II renamed Justus-Liebig Universität Giessen) in 1824 and o. Professor in 1826. There he established the first major (teaching and research) school of chemistry and the journal *Annalen der Chemie und Pharmacie* (subsequently *Justus Liebig's Annalen der Chemie*) before moving to the University of Munich in 1852. He discovered N_2, improved elemental combustion analysis and trained certain scientists named in this work: *C. von Voit*, *H. von Fehling*, *H.F.M. Kopp*, *A. Kekulé*, *A. von Hofmann*, *E. Erlenmeyer*, and *A. Strecker*.

[3] *Joseph-Louis Comte de Lagrange*, born *Guiseppe Lodovico Lagrangia*, was born on January 25, 1736 in Turin and died on April 10, 1813 in Paris. A mathematician and astronomer, he succeeded *Euler* in 1766 as director of mathematics at the Prussian Academy of Sciences in Berlin, where he remained for 20 years. In 1787, he moved from Berlin to France and became a member of the French Academy.

[4] *Friedrich August Kekulé von Stradonitz*, was born on September 7, 1829 in Darmstadt and died on July 13, 1896 in Bonn. He matriculated at the University of Giessen and, swayed by *Liebig*, studied chemistry. After postdoctoral studies in Paris (1851–1852), Chur (1852–1853), and London (1853–1855) where he came in contact with *Alexander Williamson*, Kekulé became a *Privatdozent* at the University of Heidelberg in 1856 and o. Professor at the University of Ghent in 1858. In 1867 he was called to a chair at the University of Bonn. Among other attributes, *Kekulé* is known for his theory of chemical structure, tetravalent carbon, and the structure of benzene – the last a subject of controversy. (See, for example, *Bader A* (1998) The *Wiswesser-Loschmidt* Connection. Bull Hist Chem 22:21, and references therein.) Footnote (2) of *Kekulé*'s 1865 paper [*Kekulé A* (1865) Sur la Constitution des Substances Aromatique. Bull Soc Chim 3:98] shows *Kekulé's* preference for his own structure of benzene over those of *Loschmidt* and

schaftlicher Chemie . . .” [*Lavoisier*, the true founder of scientific chemistry]. By 1859, *Kekulé* had defined organic chemistry as the chemistry of carbon compounds (*28*), but *Lavoisier*, too, may have been a founder of modern organic chemistry by his (*27*): “(i) recognition of the qualitative composition of vegetable and animal substances, (ii) recognition that these contain compound radicals which can combine with oxygen to form oxides such as sugar or alcohol and acids such as oxalic and acetic acids, and (iii) introduction of a method of combustion analysis”. Animal anatomy and physiology were becoming linked to physiological chemistry and pharmacology, or animal chemistry, which continued to attract “chemical” probing of tissues and fluids. These domains were not in the least exempt from the scientific and chemical revolution taking place in the late 18th century.

The middle of the 18th century ushered in a period of intense scientific investigation, which for chemistry involved building upon a rapidly expanding and often confusing world of empirical knowledge of chemical substances and their manipulations, and the development of laboratory apparatus. Modern chemistry was thus emerging from its roots in alchemy and medicine, from a medicinal chemistry that predated *Paracelsus*, and over time led to the preparation of drugs by distillation of all types of plant and animal sources. Nonetheless, one should not think that scientific investigations of the 1800s necessarily involved pure chemicals, especially those of biological origin.

The historic philosophical-medical interest in animal fluids: blood, phlegm, bile, and urine, led to probings beyond dry (or destructive) distillation to the mixing of chemicals with such fluids, either before or after water had been gently removed, in order to effect separation into the component parts that could be probed independently. Like many biological fluids, bile turned out to be a complex mixture, and the early investigations were understandably constrained by a lack or absence of chemical knowledge. Modestly successful investigations of the 18th century typically involved manipulations using additives such as mineral acids, acetic acid, alcohol, ether, lead and barium salts, to effect separations by combinations of sequences involving precipitations, washings, and extractions. While such efforts were of limited success for isolating the pigments of bile, they were useful in isolating the fatty substances that are typically the major components of bile and gallstones, which led investigators of bile of the 18th century to focus less often on the pigments and more often on substances we now know as cholesterol, bile salts, and fatty acids.

Crum Brown – an apparent recognition of earlier published conceptual structures of benzene. Most notably, a cyclic structure for benzene from 1861 by *Johann Joseph Loschmidt*, professor of physical chemistry at the University of Vienna, who was born on March 15, 1821 in Karlsbad (now Karlovy Vary) and died on July 8, 1895 in Vienna. [Loschmidt J (1861) Chemische Studien I. Carl Gerold’s Sohn, Vienna]. And in an unusually clear and modern molecular representation of phenol in the 1861 M.D. thesis of *Alexander Crum Brown*, professor of chemistry at the University of Edinburgh from 1869-1908, who was born on March 20, 1838 in Edinburgh, where he died on October 28, 1922 [*Crum Brown A* (1866) On the Classification of Chemical Substances. Trans Roy Soc Chem 24:331]. *Kekulé*’s famous students include *van’t Hoff*, *Emil Fischer*, *Adolf von Baeyer*, and *Richard Anschütz*.

To the anatomists and physiologists of the first half of the 18th century and earlier, bile was seen as a yellow or yellowish-green, slightly alkaline, and slimy fluid possessing a peculiar sickening odor, with a taste at first sweet then bitter and exceedingly nauseating. Of variable consistency, commonly ropy and viscid, but at times limpid, it was found to be of greater density than water and miscible in all proportions with it (*29–31*). The fact that bile was used at times as a soap suggested a composition of animal fat and alkali (*31*). The opinion of the physiologists of the era might be best summarized as expressed by the physician *Thomas Coe* in 1757 (*29*) in which the yellow color of bile is mentioned:

> That bile is of a saponaceous nature appears by a plain experiment known to the vulgar, that is the use of the gall of oxen in washing linen, scouring wool, & where, like soap, being mixed with water, it helps to wash out grease and other stains, which the water alone could have little or no effect upon. . . . The bile is of two kinds, namely that of the gall-bladder, called *bilis cystica*, and that which comes directly from the liver to the gut, called *bilis hepatica*. The cystic bile is thicker, of a deep yellow color and very intensely bitter.

And in 1767, *Cadet*[5] wrote of bile (*32, 33*):

> Je puis donc conclure que la bile est un véritable savon composé d'une graisse animale et de la base alkaline du sel marin, et du sel marin lui-même, d'un sel essentiel de la nature du sucre de lait et d'une terre calcaire qui participe un peu du fer. 1*

The color and bitterness of bile were attributed to the last two principles together with the nature of the oily principle (*32, 33*). Such was the status of animal chemistry of the times and such was its colorful terminology: sugar of milk (= lactose), calcareous earth (= CaO), and ferruginous (= rust colored).

Investigations were not limited to bile alone but were quite naturally drawn to concretions that appeared in bile, or were more generally found in the gallbladder. Again, in 1757, *Coe* described bile stones in the English scientific language of the times (*29*):

> And when by any means the bile is stopped or retarded so as to stagnate long either in the gall-bladder or ducts, especially if before the stoppage it was unusually thick or viscid, or abounded more than ordinarily with earthy particles, it is readily formed into biliary concretions, or gall-stones, of various kinds. . . . It has been observed that some of these calculi seem to be made almost solely of earthy particles, cemented together, perhaps by a kind of mucus, without any appearance of bile; and that others seem to consist of mere inspissated or thickened bile without any mixture of earth, which will be different from one another, according to the bile from which they are formed, whether it was black or yellow, or green, or of some other color; but that the greater part of them are an undoubted mixture of earthy particles and bile, as both are plainly seen in the composition. . . . [S]ome are compact and hard, and rather heavy, others are soft, or friable, and light.

For some early attempts at "chemistry":

> Biliary concretions do not dissolve in water, even with boiling, though the bile itself readily dissolves and mixes with it. Nor are they soluble in spirituous menstruum, as neither indeed does the bile dissolve well in rectified spirit, though it does in a weak spirit.

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[5] *Louis-Claude Cadet de Grassicourt*, 1731–1799, studied at the Collège de Quatre-Nations and was a pharmacist at the Hôtel Royale des Invalides in Paris.

[*] Please note that translations numbered in this manner are provided in Section 11.

However,

> . . . some large and soft ones dissolved to about half their bulk in hot water. . . . [T]hey will not dissolve in lime-water . . . but some of them will dissolve in lixivium of salt of tartar. Most of the gall-stones will burn and flame more or less when they are dry . . .

where the almost alchemical terms, spirituous menstruum = aqueous alcohol; rectified spirit (of wine) = repeatedly distilled alcohol, to concentrate; weak spirit (of wine) = dilute alcohol; lime-water = a clear solution of $Ca(OH)_2$; lixivium of salt of tartar = aqueous alkaline extract of wood ashes, or a solution of K_2CO_3, give evidence to the richness of abandoned chemical terminology.

Yet gallstones eventually proved to offer easier access than bile itself to the fatty materials contained therein, and, as shall be seen, also to the yellow and green pigments of bile. In the first half of the 18th century *Vallisneri*[6] noticed shortly before his death that gallstones dissolved in a mixture of spirit of wine (alcohol) and turpentine (*34*). And by the middle of the 18th century physicians considered gallstones to consist of the same oily, flammable material as in bile. In 1764, in his *Elements of Physiology* (*35*), the famous anatomist of his time, *Haller*[7] summarized the knowledge of gallstones at that time:

> The bile concretions contain a lot of air, up to four times their volume. Some are almost tasteless, except for the nucleus, which is bitter. They dissolve best in alkali, but fail in oil of tartar, and dissolve in potassium carbonate, for example, and very completely in oil of turpentine, sometimes in spirit of wine, sometimes it dissolves in none of these materials. They soften and dissolve in dilute nitric acid while sulfuric acid is without effect on them. Subjected to distillation, they soften and flow like sealing wax and then produce a little phlegm, a yellow oil, then a red oil and finally a black and empyreumatic oil.

Such were the early chemical investigations.

At about the same time as *Haller*'s 1764 treatise, *Poulletier de la Salle*[8], a contemporary of *Lavoisier*, practiced experiments on bile during 1745–1755, confirming that it had a soapy nature and contained an alkali salt – an observation by then well-known that was reconfirmed by *Cadet* (*32*). A more important observation was *Poulletier*'s apparent isolation of what we now know as cholesterol from gallstones. Stimulated perhaps by *Vallisneri*'s work on the solubility of gallstones, and assisted by a young *Fourcroy* in 1786–1787, from a fairly large collection of human gallbladder gallstones, *Poulletier* powdered some and dissolved them with warming in alcohol (and thereby confirmed *Vallisneri*'s experiment). Small blades of a glistening white crystalline substance appeared upon cooling, doubtless what we now know as cholesterol and the primary constituent of most human gallstones.

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[6] *Antonio Vallisneri*, 1661–1730, a professor of practical medicine in Padua.

[7] *Albertus (Albrecht) von Haller* was born on October 16, 1708 in Bern and died on December 12, 1777. He studied anatomy at Tübingen (1723–1725) and at Leiden, where he graduated in 1727, studied mathematics at Basel (1728), practiced medicine in Bern (1729) until answering a call to the University of Göttingen in 1736 and resigned his chair in 1753 to return to Switzerland.

[8] *François Paul Lyon Poulletier, Sieur de la Salle*, was born on September 30, 1719 in Lyon and died on March 20, 1788 in Paris. He was the first to isolate crystals of cholesterol in ~1758.

Poulletier apparently communicated his results to *Macquer*[9], who reported them in his *Dictionnaire de Chymie* in 1788 (*36*, *37*). If he had an interest in the pigment of gallstones, it was not evident.

Between 1775 and 1789 the shiny crystalline material from gallstones was also isolated by others (*37*): the theses of *Conradi* (*38*), *Delius* (*39*), *Dietrich* (*40*), and the compilation of *Gren* (*41*). In 1775, *Conradi* repeated *Poulletier*'s preparation, and similar experiments were carried out by *Delius* in 1782, and *Dietrich* in 1788, working with Prof. *Gren* – with the same result: isolation of a fatty substance that *Gren* called gallstone fat. *Delius*, *Dietrich*, and *Gren* gave an early analysis of gallstones: 85% waxy material; 15% resinous material (*37*). Any colored material present was not investigated. Together with *Vauquelin*[10], *Fourcroy*[11] (*42*, *43*) continued the investigations of *Poulletier* on gallstones, perhaps from the same collection. Bile stones were divided according to their external color (brown or black, yellowish or greenish, white and ovoid) and distinguished by "chemical analysis": specific gravity compared to water, exposure to a flame, dry distillation, alcohol treatment. Some were noted to have a green-brown internal color. The pulverized stones dissolved with warming in alcohol, except for the hard and brown parts, and after filtration the liquid exhibited a yellow to light green color. (This was apparently the first chemical separation of pigments from gallstones.) Cooling the alcohol extract yielded brilliant white crystals having a waxy, scaly appearance of what *Fourcroy* thought (incorrectly) to be adipocire (now known as a mixture of calcium and potassium palmitates) (*27*) and spermaciti (*37, 42, 43*). The name for the white crystals would later (1815) be given as cholestérine (Greek: $\chi o \lambda \eta$ for bile; $\sigma \tau \varepsilon \rho \varepsilon \acute{\alpha}$ for solid) by *Chevreul*[12] who showed that it was unsaponifiable (*44–46*). Investigations of the colored material of bile and gallstones would have to wait until the 19th century.

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[9] *Pierre-Joseph Macquer* was born on October 9, 1718 in Paris and died on February 15, 1784. He was one of the most famous chemists of his era and was known in particular for his *Dictionnaire de Chymie* first published in 1766, with subsequent expanded editions that followed.

[10] *Louis Nicholas Vauquelin*, was born on May 10, 1763 in Normandy and died on November 14, 1829. He was an assistant in *Fourcroy*'s laboratory from 1783 to 1791, and from 1809 Professor at the University of Paris.

[11] *Antoine François le Comte de Fourcroy*, was born on June 15, 1755 in Paris and died on December 16, 1809 in Paris. He was a physician turned chemist with help from the famous French anatomist *Felix Vicq D'Azur* (1748–1794), studied at the Faculté de Mèdicine in Paris, was promoted to chair of chemistry at the Jardin du Roi, Musée d'Histoire Naturelle upon the death in 1784 of *Macquer*, Professor of Chemistry at the Collège de France.

[12] *Michel Eugène Chevreul* was born on August 31, 1786 in Angers and died on April 9, 1889 in Paris. He lived through the "Reign of Terror" as a youth in France, applied to *Fourcroy* and worked in the laboratory under *Vauquelin*. His first teaching appointment was at the Lycée Charlemagne; in 1810 he became Assistant Naturalist at the Museum; in 1821 Examiner in Chemistry at the École Polytechnique. In 1826 he was elected to the Acadèmie des Sciences and in 1830 elected successor to *Vauquelin* as the Administrative Professor of the Musée d'Histoire Naturelle. Over such an incredibly long life he saw and accomplished much while knowing personally most of the famous chemists in Europe.

2.2 Color Diagnostics

In 1753, *Georg Heuermann* (1723–1768) wrote that yellow bile turned green in the presence of air and under the influence of acid (*47*):

> Das merckwürdigste hiebey ist, daß selbige, wie der Herr Seger schon augemercket (‘De orfu et progressu bilis cysticae, § 13′) durch beymischung des Spiritus nitri, salis und Olei Vitrioli, so besonders ihre Farbe verwandelt, denn mit dem ersten wird es *fast augenblicklich* grün… 2

The color change in yellow bile resulting from addition of nitric acid had also been observed, as recorded in *von Haller*'s 1764 treatise on physiology (*35*) in his chapter on the action of acids on bile (*ut se habeat ad acida*):

> Spiritus nitri bilem efficacius cogit, ut virides et duri grumi in aero subsideant. Viridem fecit, quae flava fuerit . . . Cum aqua forti alias arbusculae virides natae sunt; et grumus in fundo subsedit. In aliis puto meracioris acidi exemplis, bilis in coagulum amarum, viridis resinae similis, abiit… 3

A series of color changes were reported, in 1794, as having been seen by *Marabelli* when nitric acid was added to bile (*48*). Such color changes continued to be observed into the early 19th century when *Tiedemann*[13] and *Gmelin*[14] reported a detailed, systematic investigation in their famous treatise on digestion, *Die Verdauung nach Versuchen*, describing and analyzing the reaction (*48*). *Tiedemann* and *Gmelin* noted that when yellow-brown bile from a dog was treated with hydrochloric acid that had been de-aerated (freed from oxygen) no color change occurred during several days, but when oxygen was introduced the solution turned green near the oxygen inlet. They had thereby established a link between color change and oxidation/oxygenation. The color change from yellow to green was not restricted to hydrochloric acid treatment but was also observed following addition of sulfuric acid or acetic acid – and nitric acid. In the last, the color change to green was more rapid and was followed by further changes in color (in succession: green, blue, violet, red and finally yellow) in bile from mammals, birds, amphibians and fish (*48*):

> Dieselbe Wirkung, jedoch augenblicklich und weiter schreitend, zeigt die Salpetersäure, ohne Zweifel weil sie selbst den zur Farben veränderung nöthigen Sauerstoff abgiebt. Alle Arten von Galle, sowohl von Säugthieren, als Vögeln, als Amphibien und Fischen, die wir in dieser Beziehung untersuchten, färbten sich bei allmähligen Zufügen von Salpetersäure

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[13] *Friedrich Tiedemann* was born on August 23, 1781 in Kassel and died on January 22, 1861 in Munich. He studied medicine and science in Bamberg and Würzburg, earning the Dr. med. in 1804 in Marburg, while continuing studies in Paris and Würzburg. In 1805 he became professor at Landshut and in 1815 accepted a position as Professor and Director of the Institute of Anatomy at Heidelberg, for 33 years.

[14] *Leopold Gmelin* was born on August 2, 1788 in Göttingen and died on April 13, 1853. He studied medicine and chemistry at Göttingen, Tübingen, and Vienna and in 1814 was appointed a. o. Professor and in 1817 o. Professor of chemistry and medicine at Heidelberg until 1852. *Gmelin*'s *Handbuch der Chemie* was first published in 1817–1819, and many successive editions appeared as the *Handbuch der Anorganischen Chemie*.

> erst grün, dann blau, dann violett, dann roth, und zwar alles dieses bei hinreichen der Säuremenge innerhalb weniger Secunden. Hierauf tritt in einigen Stunden oder, bei grösserem Säureuberschüss, in einigen Minuten, Zerstörung der rothen Farbe ein, worauf die Flüssigkeit gelb erscheint. 4

Again, using nitric acid, the same authors detected the same progression of color changes in pathologic blood serum, chylus serum and urine, thereby indicating the presence of the pigment of bile (*48*):

> Mittelst dieses Verhältnisses haben wir den Farbstoff der Galle in krankhaftem Blut-Serum; Chylus-Serum und Urin entdeckt, und es möchte hierdurch auch eine medicinische Wichtigkeit erhalten, da es zur Auffindung der Galle das sicherste Mittel ist… 5

and citing potential medical importance to this diagnostic color test for detecting the presence of bile in other tissues.

Tiedemann and *Gmelin* reported further on color reactions of bile following the addition of chlorine and from attempts using base to probe the colors obtained during the various stages of the nitric acid reaction. They learned that although oxygen is necessary to turn yellow into green in acids such as HCl, H_2SO_4, and acetic acid, only nitric acid (and the traces of NO_2 present) is required for the spectrum of colors. The work established what became famous as the G*melin* reaction (or *Gmelin* test) for bilirubin (*48*):

> Man versetze z.B. Hundegalle mit so viel Salpetersäure, dass die blaue Färbung eintritt, übersättige sie dann mit Kali und giesse dann Vitriolöl in hinreichender Menge hinzu, so hat man ein Stück von *Regenbogen*; nämlich über dem farblosen Vitriolöl befindet sich eine rosenrothe Schicht, darüber eine blaue, dann eine grüne, und zu oberst eine gelbgrüne… 6

– the tints of the rainbow.

The *Gmelin* reaction was used for many decades following 1826 as a medical test to detect and characterize bilirubin in urine or other body tissues and over time was elaborated by the German physician *Ottomar Rosenbach*[15] (1851–1907), when it became known as the *Gmelin-Rosenbach* or *Rosenbach-Gmelin* color test (*49*). In one variation of the test, suspected urine or aqueous pigment is layered onto concentrated nitric acid (containing nitrous acid) or fuming nitric acid contained in a small tube so that it forms a layer on top. From the liquid-liquid junction outward disc-like rings are formed from the interface upward of colors yellow, red, violet, blue and green. In another variation, urine is passed through the same filter paper several times, the filter paper is dried and spotted with a drop of (slightly fuming)

[15] *Ottomar Ernst Felix Rosenbach* was born on January 4, 1851 in Krappitz, Silesia and died on March 20, 1907 in Berlin. He was educated at the universities in Berlin and Breslau (Dr. med., 1874). From 1874 to 1877 he was *Assistenzart* to *Leube* and *Nothnagel* at the medical hospital at the University of Jena, and in 1878 was *Oberassistent* at the Allerheiligen Hospital in Breslau, and became *Privatdozent* at the University. Rising to chief of the department of medicine of the hospital, he was appointed Assistant Professor in 1888, and resigned his position in 1896 to return to Berlin.

nitric acid to form a yellow spot with characteristic concentric rings of red, violet, blue and green. The colors are also reproduced in organic solvents: a yellow solution of the pigment in $CHCl_3$, treated with one drop of fuming HNO_3, becomes green and then in rapid succession blue, violet, reddish-orange and finally pale yellow or colorless.

Yet neither at the time (1826) of *Tiedemann* and *Gmelin*'s *Die Verdauung nach Versuchen*, nor until the 1840s, were the color changes shown to depend on a *specific* pigment in bile. That, of course, required some form of isolation.

2.3 Emergence of a New Analytical Methodology: Quantitative Combustion Analysis

At the end of the 18th century, *Fourcroy* summarized (*50*) the typical methods employed for analysis of organics from plant or animal products (*22*):

1. Natural mechanical analysis (separation by nature).
   Exudates of plants – saps, gums, manna, resins, rubber.
2. Artificial mechanical analysis (separation by presses, mortars).
   Juices and oils. The product is unaltered.
3. Distillation.
   Forms products which may not have been present as such in the plants.
4. Combustion analysis.
   Produces quantity of carbon and ash.
5. Analysis by water.
   a. Soaking after maceration.
   b. Soaking with agitation.
   c. Infusion (boiling water poured over macerated tissues).
   d. Digestion (tissues in cold water are heated slowly until boiling point is reached).
   e. Decoction (tissues are boiled with water for several hours).
   The various forms of analysis by water result in progressively greater alteration of the tissue components.
6. Analysis by acids and alkalies.
   Treatment may be similar to analysis by water, but alteration of principles is generally greater.
7. Analysis by alcohol, ether or oils.
   Results in a selective dissolving action; i.e., alcohol dissolves essential oils but no fixed (fatty) oils.
8. Analysis by fermentation.

As *Ihde* wrote (*22*):

> As is clearly evident, the above analytical procedures are, at best, capable only of separating mixtures of related substances (proximate principles). Frequently the separation is achieved only after significant chemical alteration. The analyses could have only superficial value in leading to an understanding of organic materials; in many instances they were downright misleading. The time was becoming ripe for a more sophisticated approach, one which demanded pure, unaltered compounds which could be analyzed for their component elements, and studied for their characteristic properties.

To these "analytical procedures", now deservingly absent from organic chemistry, one might add dry distillation (heating a solid, often absent air, to produce and remove gaseous products) and, similarly, calcination, a method in which a substance is heated in air to a high temperature in a crucible to drive off water, carbon dioxide, and other volatiles until it is reduced to ash, which is then analyzed (see #4, above). Such methods of analysis date back to alchemy; yet, as will become clear in the attempt to analyze bile and gallstones, they had not been abandoned entirely in the 1800s.

With its roots in the very late 18th century, a new and revolutionary technique for organic analysis had, within a few decades of its discovery, reached a useful level of reliability and offered something no other previous method of analysis could: an empirical formula for the (presumed) pure substance. Thus, *Lavoisier*'s novel 1794 method (*24*) for analyzing the composition of alcohol, fats and waxes by combusting them and measuring the oxygen consumed and CO_2 produced, although yielding results that were usually inaccurate, opened the door to improvements in combustion techniques and gasometric measurements. As elaborated by *Holmes* and *Levere* (*51*), the rather large and cumbersome *Lavoisier* device was followed fairly rapidly by improvements: (i) in 1810 by *Gay-Lussac*,[16] working with *Thenard*, who upgraded the combustion process by admixing $KClO_3$ with the sample, then later abandoned it in favor of admixing CuO, for reasons of safety; and (ii) between 1811 and 1815 from *Berzelius* working with *Gay-Lussac* who in 1815 reintroduced the use of $KClO_3$, but admixed with NaCl to temper the combustion; and (iii) *Liebig*, working between 1822 and 1824 with *Gay-Lussac*, who replaced bell jar gasometry with the combustion train (the *Kaliapparat*) wherein water vapor formed by the combustion of a weighed sample was absorbed by $CaCl_2$ and CO_2 was absorbed by KOH (*Kali*), both being weighed. Thus, in the early 19th century a major contribution to scientific methodology had come about in the technique called combustion analysis that enabled one to determine the %C and %H (and ash) in organic or biological samples from a quantitative measure of the CO_2 and H_2O produced (*22*, *49*, *50*). This methodology was followed shortly by one developed to determine the %N (*22*, *49*, *50*), a particularly major advance, as one could begin to group biological substances according to whether they contained nitrogen – and how much. Consequently, in the 19th century scientists were able to perform certain analyses involving partial separations of components of a mixture, probe the mixture and its components by treatment with chemicals such as acids and bases and heavy metals, alcohol and various organic solvents, and combust the components in order to obtain a quantitative measure of their %C, H, and N, with an eye toward calculating an empirical formula.

[16] *Joseph Louis Gay-Lussac* was born on December 6, 1778 in Saint Léonard de Noblat and died on May 9, 1850 in Paris. He was assistant to *Berthollet* and demonstrator to *Fourcroy* at the École Polytechnique in Paris, and became professor of chemistry in 1809. From 1808 to 1832 he was professor of physics at the Sorbonne, in 1832 chair of chemistry at the Jardin des Plantes. He is best known for his two gas laws and recognition of iodine as an element.

Improvements to the determination of %N were advanced by *Dumas* and *Kjeldahl* during the 19th century, and the development of modern-day organic microanalysis was advanced by *Pregl*[17] in Graz, who demonstrated early in the 20th century that quantitative analysis for C, H, N, S, and halogens could be accomplished with 7–13 mg of a sample, then down to 3–5 mg, with weighings ±0.001 of a milligram and the accuracy of macroanalysis. Until the advent of modern spectroscopic methods, elemental combustion analysis and microanalysis became fundamentally important to understanding organic structure. *Pregl*'s contributions, honored with a *Nobel* Prize in 1923, were probably the most important advance in organic analysis following the time of *Liebig*.

2.4 Early 19th Century Pigment Separation from Bile and Gallstones

As the 18th century drew to a close, and *Napoléon Bonaparte* (1769–1821) of France emerged to dominate the European continent in war and in law during the first decade and a half of the 19th century, organic chemistry was very much still the chemistry of animal and vegetable components, largely a descriptive science oriented toward the isolation and identification of the products of nature. Destructive distillation (calcination), which had served for centuries, was being abandoned as an analytical method and replaced by new approaches aimed at isolation of components in a state unchanged by the process of separation.

The revolution in scientific thought and experimentation of the 18th century thus brought into the turbulent 19th a new perspective in organic chemistry, which was still steeped in "Vitalism" but poised to broaden into the realm of interconversion and synthesis in a laboratory environment. For the animal and plant chemistry precursors to organic chemistry were, fewer than 200 years prior to this writing, clearly natural products, and vitalism was a widespread belief that organic compounds were to be found only in animal or plant sources, produced there by a "vital force" until *Wöhler*[18] overthrew ancient dogma (Vitalism) by creating an organic substance (urea) from its elements by heating an inorganic source (NH_4CNO, ammonium

[17] *Fritz Pregl* was born on September 3, 1869 in Ljubljana and died on December 13, 1936 in Graz. An Austrian chemist and physician, he received the Dr. med. degree in 1894 at the University of Graz, studied in Germany in 1904 with *Gustav v. Hüfner*, in Tübingen, *W. Ostwald* in Leipzig, and *E. Fischer* in Berlin before returning to work at the Medico-Chemical Institute under *K.B. Hofmann* at the University of Graz. He was appointed forensic chemist at Graz and professor at the University of Innsbruck from 1910 to 1913 before being recalled to Graz in 1913.

[18] *Friedrich Wöhler* was born on July 31, 1800 in Eschersheim and died on September 25, 1882 in Göttingen. He studied under *Gmelin* in Heidelberg and *Berzelius* in Stockholm, taught chemistry at the Gewerbeschule in Berlin from 1825, in Kassel from 1831, and in 1836 he became o. Professor of Chemistry in the medical faculty at Göttingen.

cyanate) (*52*). That event contradicted the firm beliefs of respected scientific authorities, such as *Gmelin*,[19] who said in 1817 that a characteristic of organic compounds was that they could not be produced from their elements; and *Berzelius*, who in 1827 believed that the elements present in living bodies obeyed laws totally different from those that rule inanimate nature.

The early 1800s were clearly a lively period for chemical science, with new discoveries occurring at a rapid rate. In the spirit of the Enlightenment, it was also a contentious period where firmly held beliefs were being challenged and reinterpreted or discarded, often reluctantly. Nonetheless, it ushered in a quantitative analytical method (combustion analysis) important during the following two centuries for determining not only the elemental composition but also the empirical formula of a sample, no less for bile pigments. And though the characteristic yellowish and greenish colors associated with bile had been recognized for millenia, it was not until the first half of the 19th century that modestly successful separations of the pigments from their biological sources were achieved. The typical source targets were well known from their yellow color: urine, bile, gallbladder and gallstones. Bile and gallstones were the most intensively investigated; the first turned out later to be the poorest source, the latter the best. Thus, early in the 19th century, three famous scientists, *Thenard* in France, *Berzelius* in Sweden, and *Gmelin* in Germany, commenced their chemical analyses of bile and gallstones – although not specifically to isolate the coloring matter.

Late in the first decade of the 19th century, when during the Napoleonic wars the Holy Roman Empire of 234 states was dissolved in 1806 and replaced in the Congress of Vienna in 1815 by the German Confederation of 39 states, the English Romantic poets *George Gordon Lord Byron* (1788–1824), *Percy Bysshe Shelley* (1792–1822), and *John Keats* (1795–1821) began to produce their famous literary contributions. And *Thenard* and *Berzelius* began to report the first chemical studies of bile and its concretions. In 1807 *Thenard*[20] reported his results from undertaking an analysis of the bile of several animals (*53–56*). In his bile analysis *Thenard* used reagents not previously employed, including acetic acid and lead oxide to effect precipitation and thereby initiated separation. When treating yellow-green bile from an ox gallbladder with H_2SO_4, HNO_3, or HCl, in all cases a yellow material was formed, along with little resin. Using, variously, alcohol, ether, $BaCl_2$, lead acetate, from the precipitated barium or lead salts and the supernatant he obtained

[19] *Jöns Jakob Berzelius* was born on August 20, 1779 in Väversunde and died on August 7, 1848 in Stockholm. He was perhaps the most influential scientist of the first half of the 19th century, and one of the fathers of modern chemistry, graduated as a Dr. med. in 1802 in Uppsala, became assistant professor of botany and pharmacy at Stockholm and full professor in 1807. From 1815 to 1832 he was professor of chemistry at the Karolinska Institute in Stockholm. Early on, his interests were physiological chemistry but expanded rapidly to include the law of definite proportions, and he compiled tables of relative atomic weights, or atomic equivalents, *etc*.

[20] *Louis Jacques Thenard* was born on May 4, 1777 in La Louptière and died on June 21, 1857 in Paris. He was the son of a peasant, became *Vauquelin's* laboratory boy in Paris at age 17 and was helped by *Fourcroy* to succeed *Vauquelin* in the Collège Polytechnique (1804–1837) as professor, where he worked with *Gay-Lussac* (also a professor from 1809) and became famous for his discovery of hydrogen peroxide in 1818.

yellow material and, respectively, three essential principals: soda, a resin, and a substance that he named *picromel* (a colorless, viscous substance having a bitter-sweet taste). As *Thomson* wrote (*30*): "The name picromel is, I presume, from $\pi\iota\kappa\rho o\zeta$: bitter, and $\mu\varepsilon\lambda\iota$: honey." The substances were later shown to be mixtures: with picromel containing principally salts of bile acids that later became known as glycocholic acid and taurocholic acid.

The biles of many different animals were analyzed by *Thenard*, including that of the ox and humans. After evaporation of 800 parts of ox bile to dryness, and calcining; or 1,100 parts of human bile, quantitation showed:

Composition	Ox	Human
Water	700	1000
Albumin		42
Picromel	60.3	
Resin	24	41
Yellow matter	4	2-10
Soda	4	5.6
Phosphate of soda	2	
Muriate of soda	3.2	
Sulphate of soda	0.8	4.5
Phosphate of lime or perhaps magnesia	1.2	
Oxide of iron	trace	

Thenard found that human and quadruped bile contained similar substances, that the resin was sometimes green and sometimes yellow, that while human bile is sometimes green it is nearly always yellow-brown – and at times colorless. He noted that the yellow material from human bile and ox bile was insoluble in water and in kerosene but soluble in alkali – and that the alkaline solution upon acidification with HCl formed a flocculent green-brown precipitate (*54*). *Thenard's* research into bile extended to include that from fish, birds, *etc*.

Thenard also investigated gallstones as well as bile, from humans and from cattle. He found the concretions or calculi "absolument sans saveur et sans odeur", without taste or odor, and that the color was always yellow. When the yellow stones were exposed to air they gradually went green. When the stone was dissolved in caustic alkali, a yellow solution was obtained that gave a green precipitate upon addition of acid. He cites *Poulletier*'s crystals obtained from human gallstones by partially dissolving in alcohol and concludes, with *Fourcroy*, that the stones have yellow lamina with a yellow interior and that they contain 88–94% *adipocire*. Ox gallstones exhibited brown-black coloration and contained variable yellow material. It might thus be said that *Thenard* had performed the first crude partial separation of the components of bile and gallstones, into yellow and green components, *inter alia*, and that he had noticed the yellow coloring undergoing a change to green from exposure to air.

Simultaneously, *Berzelius*, who later became virtually the supreme authority in Europe on matters of chemistry, had initiated investigations of bile, which he reported to the Swedish Academy in 1806–1808 (*57*). This study, previously reported in Swedish and perhaps not read widely, was communicated to the Royal Society of Medicine in England, by invitation. Some of his principal results were thus (*58*):

1. *Of Bile*.

It is well known that the elder chemists considered the bile as an animal soap composed of soda and a resin. The accuracy of this opinion had often been questioned, owing to the very small proportion of soda; and lately our skilful contemporary Thenard, has published an analysis of bile, in which he gives as its component parts, soda, a peculiar matter name by him *Picromel* and a resin, which united, produce a fluid that has the taste and other distinguishing properties of this secretion. Nevertheless I am convinced that there is no such resin as Thenard and his predecessors have described. I shall not here relate my experiments on this supposed resin in particular, but shall give the results of my enquiries on the bile itself, which will enable the reader to confirm or reject my opinions according as he finds them founded on accurate experiment.

The substance which is peculiar to bile has an excessively bitter taste followed by some sweetness; the smell is also peculiar, and the colour in most animals varies from green to greenish yellow. It is soluble in water, and its solubility is not in the least promoted by the alkali of bile, since, when this is neutralized by an acid, the peculiar matter does not separate: it also dissolves in alcohol in all proportions. Like the albuminous materials of the blood of which this peculiar matter is composed, it will unite with acids, producing compounds of two degrees of saturation, and hence, of solubility. The acetous acid, which gives soluble compounds with the albumen of the blood, does the same with the peculiar matter of the bile; and hence this matter is not precipitated on adding this acid to bile, though it falls down on the addition of the sulphuric, nitric, or muriatic acids. It is this sparingly soluble compound of biliary matter with a mineral acid which has been mistaken by chemists for a resin; since it possesses the external characters of a resin, melts when heated, dissolves in spirit of wine, and is again precipitated (in part at least) by the addition of water. The alkalies, alkaline earths, and alkaline acetates decompose and dissolve it: the former by depriving it of its combined acid; the latter, by furnishing it with acetous acid which renders it soluble in water. . . .

The biliary matter may be obtained pure in the following way: mix fresh bile with sulphuric acid diluted with 3 or 4 times its weight of water; a yellow precipitate of a peculiar nature first appears, which must be allowed to subside and be removed; then continue to add fresh acid as long as any precipitate is formed; heat the mixture gently for some hours, and afterwards decant the fluid part, and thoroughly edulcorate the green resin which is left. This resin reddens litmus, and is partially and sparingly soluble in water. It may be deprived of its acid in two ways: one of them is by digesting it with carbonate of baryates and water, whereby the carbonate is decomposed, and the water forms a green solution possessing all the peculiar characters of bile: the other way is by dissolving it in alcohol and digesting the solution, either with carbonate of potass or carbonate of lime till it no longer reddens litmus, and then evaporating it to dryness. Either of these methods will give the pure biliary matter, and there are also other ways of obtaining it, which I have described in my work on Animal Chemistry, Vol. II. p. 47. [*57*]

This peculiar biliary matter when pure, resembles exactly entire desiccated bile. Being soluble in alcohol it might be supposed that it would dissolve in ether, but this is not the case, for ether only changes it to a very fetid adipocirous substance, exactly as it acts upon the albuminous matter of the blood. One circumstance relating to the biliary matter has much surprised me, which is, that it gives no ammonia by destructive distillation. Therefore it contains no azote; but what can have become of the albuminous matter of the blood? for, no vestige of azote is found in any other of the constituent parts of the bile, nor does bile contain any ammonia.

The following is the result of my analysis of bile.

Water		907.4
Biliary matter		80.0
Mucus of the gall-bladder, dissolved in the bile	}	3.0
Alkalies and salts (common to all secreted fluids)	}	9.6
		1000.00

Clearly, *Thenard*'s investigations of bile had not gone unnoticed. Though *Berzelius*' studies were contemporaneous, he employed a somewhat different separation method, relying on H_2SO_4 (not at all on acetic acid) and barium salts, especially $BaCO_3$ and heat. In this report, he strongly disputed resin matter and believed that it and the yellow matter and picromel were one and the same, merely modifications of the same substance, to which he later gave the descriptive name *Gallenstoff* (constituent of bile). He also disputed the presence of human albumin in bile, as reported by *Thenard*, for albumin is not precipitated upon addition of acetic acid, or alcohol. It is interesting to note that the 1812 synopsis (*58*) and the English or German translations of the original Swedish (*59*, *60*) differ somewhat, suggesting that *Berzelius* had not ceased work on bile since his Swedish reports in 1806–1808.

Nor had *Thenard* ceased investigations. He indicated that according to his scientific investigations, specific yellow pigments were characteristic of bile, and that pigments also occurred in large quantities in gallstones (*53–56*), thereby linking the yellow pigment to bile and concretions found in the biliary tract or gallbladder. As reported in 1827, his subsequent examination of the biliary tract of an elephant (elephants do not have gallbladders) that had died in the Paris zoo revealed dilated bile ducts that were packed with yellow "magma", yielding 500 g of a powdery yellow, water insoluble material after drying. Treatment with hydrochloric acid immediately gave a strong green color (*61*, *62*). As written from the perspective of 1977 by *Watson*,[21] an esteemed physician and porphyrinologist who studied under *Hans Fischer* in 1931–1932 (*62*):

[21] *Cecil James Watson* was born on May 31, 1901 in Minneapolis, Minnesota and died there on April 14, 1983. In 1919, he began his undergraduate studies at the University of Minnesota and entered the University of Michigan Medical School in 1921. Returning to the University of Minnesota in 1922, he completed the Dr. med. degree in 1926, after which he began a fellowship in pathology, and earned a Ph.D. in pathology in 1928. He then spent two years as the resident pathologist and director of laboratories in a private clinic in Minot, North Dakota. His interest in bile pigments came apparently from his suffering a bout of catarrhal jaundice (epidemic viral hepatitis) while in medical school, during which he made detailed observations on the course of his disease and found that urobilinogen (from intestinal reduction of bilirubin) disappeared from his excreta at the height of the jaundice – but reappeared in urine as the condition improved. Apparently, this personal experience led to his research interests in bile pigments, and it lured him back into an academic career. Returning to Minneapolis in 1930, while taking an advanced course in organic chemistry he was awarded a fellowship to study in *Hans Fischer*'s laboratory at the Technical University of Munich, where he succeeded in crystallizing stercobilin from human feces, proved the structure of stercobilinogen and showed it was not identical to urobilinogen or

> Thenard drew the curious conclusion that his green was due to impurities derived from the mucus of bile, apparently quite unaware that the HCl had converted yellow to green. From his descriptions, it seems likely that the elephantine orange pigment was a relatively pure unconjugated bilirubin, quite analogous to the pigment calculi of cattle and those so relatively common in the human bile ducts in India and the Orient. What a gold mine this elephant, at least for its time, and what a golden opportunity for Thenard!

Although *Thenard* appeared to be unaware that it was the yellow substance which had been converted to green by the action of acid, others had conducted scientific probings much earlier that consisted of observing and recording color changes brought about by adding reagents such as mineral acids to bile and urine. These early experiments were followed much later by attempts to isolate the colored matter – and purify it. For a description of efforts to separate the components of bile, from an early 19th century perspective, see *Thomson*, 1817 (*30*).

At nearly the same time, two well-respected Heidelberg professors of anatomy and physiology (*Tiedemann*) and of medicine and chemistry (*Gmelin*) jointly published their results on bile and, significantly, the cascade of colors following addition of HNO_3, in their famous treatise on digestion *Die Verdauung nach Versuchen* (*48*). Here they noted that a characteristic, very distinctly colored material is present in all bile, as *Fourcroy*, *Berzelius*, *Thenard*, *etc.* had seen earlier, and they reported achieving a partial separation (*48*):

> Schon Fourcroy nahm einen färbenden Bestandtheil der Galle an, und wiewohl es durch einige spätere Versuche zweifelhaft gemacht schien, ob eine eigenthümliche Materie der Art existire, sofern die Färbung der Galle zum Theil dem Gallenstoff zugeschrieben wurde, so hat doch Thenard *) [*) Traité de chimie edit. 4. Tom. 4. p. 580.] angenommen, dass in der Galle fast aller Thiere eine eigenthümliche gelbe Materie existirt. Er nimmt an, dass dieser Farbstoff die Gallensteine der Ochsen gänzlich constituirt und in fast allen der Menschen enthalten ist. Dieser Ansicht müssen wir uns, nach unsern Versuchen, vollständig anschliessen. Dass wirklich ein eigenthümlicher sehr ausgezeichneter färbender Körper in der Galle aller Thiere vorkomme, beweist Folgendes:
>
> 1) Wäre der Schleim das färbender Princip, so müsste, wenn man die zur Trockne abgedampfte Galle mit Weingeist auszieht, alle Farbe im unauflöslichen Schleimrückstand bleiben, wovon aber gerade das Gegentheil erfolgt. Schlägt man den Schleim durch Säure nieder, so reisst dieser zwar eine etwas grössere Menge des Farbstoffs mit sich nieder, die grösste Menge desselben bleibt jedoch gelöst.
> 2) Alle übrige Bestandtheile der Galle besitzen noch weniger Farbe, und können deshalb noch weniger als das färbende Princip derselben betrachtet werden.
> 3) Der Gallenfarbstoff zeigt, z. B. wie er in der Galle vorkommt, höchst auffallende, bis jetzt noch nicht hinlänglich bekannte Reactionen, die ihn überhaupt von allen bekannten Materien unterscheiden. Zu den wichtigsten gehören folgende.

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to mesobilirubinogen, and identified mesobiliviolin. The studies in Munich with *Fischer*, and with *Friedrich von Müller* at the medical clinic at the University of Munich, made him well situated to assume a brilliant academic career at the University of Minnesota Medical School, to which he returned in 1932. In 1934 he was appointed assistant professor of medicine, associate professor in 1936, and as professor and head of the department of medicine in 1942. After 24 years as head, he resigned in 1966 to assume research full time. *Watson* was elected to the U.S. National Academy of Sciences in 1959, authored more than 350 research publications, and during his tenure at Minnesota introduced *Fischer*-based science to the fields of bile pigments and porphyrins in U.S. medicine.

> Versetzt man die gelbbraune Galle des Hundes mit Salzsäure. . . .
>
> Die Galle der Thiere besitzt je nach ihrer Art und Individualität eine verschiedene Farbe; sie ist bei den Hunden gewöhnlich gelbbraun, nur wenig grün; bei den Ochsen braungrün; bei den Vögeln meistens lebhaft smaragd- oder grasgrün. Es lassen sich hieraus wahrscheinlich Schlüsse machen auf den mehr alkalischen oder mehr sauren Zustand der Galle, und auf den mehr desoxydirten oder mehr oxydirten Zustand des Farbestoffs derselben. 7

Gmelin also noted that gallstones contain the pigment of bile and give the same color test (*Gmelin* reaction) with HNO_3. He achieved a separation of pigmented material from pulverized ox gallstone by a series of chemical manipulations: (i) first heat in alcohol; (ii) then heat the residue in ammonium hydroxide (which gives a strong yellow color to the ammonia solution which then goes green in air); (iii) dissolve most of the undissolved residue from (ii) in aq. potash to give a yellow-brown solution that goes green-brown overnight and gives a positive *Gmelin* reaction; (iv) addition of HCl precipitated green flakes copiously from the solution in (iii). *Gmelin* carried out some further experimentation with the green flakes and concluded that the (yellow) pigment of bile and gallstones is converted to green by oxidation (*48*):

> 4) Wir untersuchten auch den Gallenstein eines Ochsen. Er liess sich leicht zu einem lebhaft braunrothen Pulver zerreiben. Kochender absoluter Weingeist färbte sich damit sehr blassgelb, nahm jedoch nur etwas festes Fett auf, welches sich nicht krystallinisch erhalten liess; als man auf den Rückstand Ammoniak einwirken liess, so nahm dieses eine etwas stäkrere Färbung an, und gab eine Flüssigkeit, die anfangs gelb war, sich jedoch an der Luft grasgrün und mit Salpetersäure blassroth färbte und durch Chlor entfärbt wurde.
>
> Der grösste Theil des Pulvers war ungelöst geblieben und dieser löste sich bei fortgesetzter Digestion mit Kali, mit Ausnahme einiger Flocken von phosphorsaurem Kalk, völlig darin auf. Diese Auflösung war anfangs gelbbraun und wurde über Nacht ebenfalls grünbraun. Sie gab mit Salpetersäure die oben bemerkten Farben-Veränderungen; sie gab mit Salzsäure einen reichlichen Niederschlag in dunkelgrünen Flocken, und nachdem sich diese völlig gesetzt hatten, so zeigte sich die überstehende anfangs noch grüne Flüssigkeit sehr blassgelb gefärbt. Die hiebei niedergefallenen grünen Flocken gaben nach dem Trocknen mit Salpetersäure eine blassrothe, bald gelb werdende Auflösung. Sie lösten sich in concentrirter Salzsäure vollständig mit smaragdgrüner Farbe; diese salzsaure Lösung trübte sich nicht mit Wasser, färbte sich mit Ammoniak gelb, mit Salpetersäure roth und wurde durch Chlor entfärbt. Auch in Ammoniak lösten sich die durch Salzsäure aus der Kali-Lösung gefällten grünen Flocken sehr leicht mit grasgrüner Farbe auf. Der Farbestoff der Galle scheint daher durch die Oxydation an der Luft, die er in der kalischen Lösung erleidet, in Salzsäure und Ammoniak löslich gemacht zu werden.
>
> Diesen Versuchen zufolge möchten wir den von uns untersuchten Gallenstein als fast reinen Farbestoff der Galle ansehen, dem nur eine kleine Menge von Fett und Kalksalzen, vielleicht auch etwas Schleim, beigemengt war, und wir möchten diesen Farbestoff wegen seines Stickstoffgehalts zunächst dem Indig setzen. 8

Although never described as pure compounds, the yellow and green material behaved like what we now know as bilirubin and biliverdin.

The pigments of bile notwithstanding, it was evident that bile was not composed of pigmented material alone but also contained fatty or soap-like components. Indeed, bile had long been considered to be a “soap”, and the soapy or lipid-like substances were attracting the attention of *Gmelin*, *Berzelius*, and other investigators, including *Demarçay* (*63*) and *Loir* (*64*, *65*).

In the period following *Thenard*'s and *Berzelius*' early investigations on the composition of bile, especially by the 1840s, a number of new investigators had independently joined the quest. While *Thenard* probably published his last findings in 1827 (*61*), *Berzelius*' investigations of bile continued well past his 1806/1808 initial studies (*57–60*), probably until he reached infirmity. Though *Berzelius*' work on bile does not qualify as his most important, it was highly advanced for its time, and thorough. *Berzelius* died in 1848, some nine years before *Thenard* and nearly 40 years past his first published studies of bile. He was apparently in declining health, as noted in his August 1839 correspondence with *Liebig*, in which he wrote of health problems (gout or arthritis) and having to take "the waters" at Marienbad as a palliative (*66*). (He apparently took seriously ill during 1818–1820 when he also had periodic head pains, then rebounded from such but again began to suffer in 1834 from the earlier nervous disorder. He suffered variable health onward, especially the two to three years prior to his death.) Despite his problems, *Berzelius* did not fail to describe his analysis of bile as not yet completed and commented on *Demarçay*'s[22] "transformation" of bile into taurine and *Gallenharz* (bile resin) as completely correct. *Berzelius* then went on to indicate that he, too, had obtained cholic acid, by his own method, and that *Demarçay*'s *acide choleïque* was an artifact that could never be obtained as the same material twice and contained at least four different organic substances. He commented that *Demarçay*'s *acide choleïque* contained two resinous acids that are also contained in his *acide choleïque*, as well as a neutral resin. *Berzelius* wrote that the major component of bile is his old *Gallenstoff*, which he now called *Bilin*, and that *Thenard*'s *picromel* and *Gmelin*'s *Gallenzucker* are not only non-acidic but are so extraordinarily sensitive as to defy purification. Writing in August 1839 to *Liebig*, *Berzelius* noted that bile also contained an acidic component, which he complained had cost him much pain to separate, and, although he had found at least four different compounds, he could not yet say which are transformation products and which are not (*66*):

> Meine Analyse der Galle betreffend, so bin ich auf lange nicht damit fertig. Am 22 Juni wurde ich von einem drohenden Gicht-Anfall auf dem Kopfe heimgesucht, der doch leicht und ohne Folgen gehoben wurde. Ich musste aber auf das Land gehen und Marienbader-Wasser trinken. Wegen meinen Amtsgeschäften musste ich 2mal in der Woche in der Stadt seyn, und diese Tage wollte ich einige Zeit die Versuche fortsetzen. Aber die warme Jahreszeit, die verdammten Fliegen die sich in meinen Auflösungen, aller Sorge sie abzuhalten unerachtet, immer ertränkten, und ein mit den Jahren schlechter werdendes Gedächtniss haben mich veranlasst alles liegen zu lassen, bis ich im October in meiner Wohnung in der Stadt wieder einziehe und mich wieder täglich damit beschäftigen kann. Demarçay's Metamorphose der Galle in Taurin und Gallenharz ist vollkommen richtig, Cholsäure habe ich auch nach seiner Methode bekommen. Es geht sogar besser mit kohlensaurem als mit caustischem Kali. —· Seine acide choleïque ist ein Kunstprodukt, das man nie zwey mal gleich erhalten kann. Es enthält wenigstens 4 verschiedene org. Substanzen, seitdem man die unorganische Säure, womit es niedergeschlagen ist, abgeschieden hat. Die acide choloïdique enthält zwey harzartige Säuren, welche auch in der ac. choleïque

[22] *Marc-Horace Demarçay*, 1813–1866, worked in *Liebig*'s lab, independently on bile for six months in the period 1836–1837.

> enthalten sind und ein indifferentes Harz. Der Hauptbestandtheil der Galle ist mein alter Gallenstoff, den ich Bilin nennen will. (Thénard's Pikromel, Gmelins Gallenzucker), der nicht sauer ist und der sich mit der grössten Leichtigkeit metamorphosirt, so dass es äusserst schwierig ist ihn rein zu bekommen. Er ist in Wasser und Alkohol in allen Verhältnissen auflöslich. Aber die Galle enthält auch sauer Bestandtheile. Es sind eigentlich diese die mir so viele Mühe gekostet haben auszuscheiden, und obgleich ich wenigstens 4 verschiedene bekommen habe, so kann ich doch in diesem Augenblick nicht sagen welche Metamorphos-Produkte sind und welche nicht. Aus einer alten *Bilis bubula spissata*, wo das Bilin grösstentheils metamorphosirt ist, kann man sie leicht ausscheiden, und auf diese Weise habe ich sie kennen lernen, aber aus der frischen Galle hält es schwierig sie hervorzuziehen, weil sie immer mit Bilin verbunden masquirt sind. Keinem von diesen gleicht die kristallinische Substanz, die Du so gut warst mir zu schicken; es kommt darauf an wie sich diese zu den Basen verhält, was ich versuchen werde. — 9

Just a few months earlier, on May 10, 1839, *Berzelius* had written to *Liebig* that *Demarçay*'s results had caused him to revise his analysis, that they reminded him of an idea he had from his own work showing that the *Gmelin* components of bile were all due to metamorphosis of the native components; that *Demarçay*'s new acid, *acide choleïque* was also a metamorphosis product, transformed by the isolation procedure (*66*):

> Demarçay's Versuche über die Galle veranlassten mich zu einer Revision der Analyse der Galle; Sie werden sich erinnern dass ich in meiner Chemie die Idé ausgesprochen habe dass die Gmelin'schen Bestandtheile der Galle alle Metamorphosen sind, was nun Demarçay bewiesen hat, aber auch Demarçay's neue Säure, Acide choleïque, ist ein Metamorphos-Produkt. Es war aus seiner Abhandlung klar, weil die neue Säure durch Essigsäure sich aus der Galle nicht ausscheiden lässt, wohl aber aus ihren Verbindungen mit den Alkalien. Es ist mir geglückt den Farbstoff der Galle auszufällen und rein zu bekommen, die Basen der Galle auszuscheiden und eine in Wasser und Alkohol in allen Verhältnissen lösliche, intensiv bittere Säure zu bekommen, die in Wasser aufgelöst von Schwefelsäure oder Salzsäure sogleich in Demarcay's Acide choleïque verwandelt wird. Sie hat die Eigenschaft Fett aufzulösen in noch höherem Grade als Seife, und wäre sie in Ether löslich, würde man sie nie vom Fett scheiden können. Sie gibt lösliche Verbindungen mit allen bis jetzt versuchten Basen, sogar mit Silberoxyd. Sobald ich mit meinen Versuchen fertig werde, werde ich für Ihre Annalen meine Arbeit mittheilen. Ich nenne die neue Säure Gallensäure, Acidum bilicum. — In einer inspissirten Galle von einer Apotheke fand ich eine ganz neue, kristallisirende Säure als Hauptbestandtheil. Ich weiss aber noch nicht ob sie in allem alten Gallen-Extract enthalten ist. Sie scheint in der Stelle der frischen Gallensäure aufgetreten zu seyn. 10

It would seem that *Berzelius* could not have commented in as much depth on the non-pigmented components of bile in 1839 unless he had continued working on them before and during the 1830s. And although he was clearly responding to *Liebig*'s comments regarding the work of *Demarçay*, he was apparently not impressed or startled by *Liebig*'s comment to him in a letter of February 1837 regarding his student, *Demarçay*, to the effect that *Demarçay*'s then recent findings on bile seemed to overturn all previously accepted results. Yet, he could not have responded as he did unless he had known some of the details of *Demarçay*'s work, published in 1838 (*63*).

> Ein fünfter (Demarçay) hat eine grosse Arbeit über die Galle vor, seine Resultate sind noch zu unbestimmt, als dass sich etwas davon mittheilen liesse, obwohl er seit 6 Monaten damit arbeitet, allein wie es scheint, so wird alles seither Angenommene umgeworfen werden. 11

Although *Demarçay*'s investigation of bile focused on the separation of what we now know as bile acids, he was not fully aware that he was working with conjugates and bile salts – and he was clearly not alone in his probing the fatty substances of bile. The advantage he had while working for six months in *Liebig*'s lab in Giessen was that he had access to *Liebig*'s state of the art combustion analysis apparatus, an advantage not available to workers in other labs where the methodology was more primitive, even if one had access to it. He published his work in 1838 (*63*), writing that bile was essentially 90% a soap, with sodium, and indicating the *Gmelin* had obtained 22 different substances from bile, almost all neutral and unknown. In Giessen, while noting the green colors of bile along the way, *Demarçay* probed ox bile variously with hydrochloric acid, ammonia, alcohol and lead salts, *etc*. so as to separate out acidic substances that the called *Choleinsäure*, *Choloidinsäure*, *Cholsäure*, *etc*. – all bile acids – as well as *Gmelin*'s *Taurin* (*63*):

> Ich gehe nun zur Beschreibung der eigenthümlichen Säure in der Galle über, welche ich *Choleinsäure* (von χολη, Galle) nenne und zu der ihrer drei Zersetzungsprodukte: der festen, stickstofffreien Substanz, welche ich *Choloidinsäure* (von χολειδηζ, gallenähnlich) nennen will, des *Taurins*, und der krystallisibaren, in Aether löslichen Säure, für welche ich den Namen *Cholsäure* beibehalten habe, denn es ist, wie ich glaube, der nemliche (?) Körper, welchen Gmelin unter diesem Namen beschrieben hat. 12

Demarçay's published work may have created more excitement than its intrinsic worth warranted. Indeed, he did publish combustion analysis results (%C, H, N) for his bile acid isolates, which were doubtless inhomogeneous. His work yielded empirical formulas such as $C_{21}H_{33,5}NO_6$ for *Choleinsäure* and even what he referred to as "atomic weights" of ~5,000, determined by burning the sodium salt of the substance and titrating the equivalents of base with acid.

Taurine, isolated in 1826 from bile first by *Gmelin* (*48*), was crystallized, apparently to purity, by *Demarçay* in beautiful needles, and its combustion analysis yielded $C_4H_{14}N_2O_{10}$, thought then to be a di-salt with ammonia. Taurine is still known today as a major component of bile, but with the formula $C_2H_7NO_3S$ ($^{-}O_3SCH_2CH_2NH_4^{+}$). Considering that in the *Demarçay* combustion analysis the percent oxygen was doubtless calculated by difference, after determining the %C, H, and N, one can imagine a *Demarçay* empirical formula $C_2H_7NO_5$, or $C_2H_5NO_3S$.

Keeping in mind that these studies represented "state of the art" organic chemistry of the late 1830s, the advances in analysis employed represented a move toward modern technology, including the use of crystallization as a means of purification to homogeneity and the emergence of combustion analysis as a powerful analytical tool.

2.5 Bilirubin and Biliverdin Separation from Bile by the Middle 19th Century

Despite the rather halting and somewhat controversial progress in isolating the yellow and green pigments identified with bile and gallstones in the early 1800s, significant headway had been made by the middle part of the 19th century. Yet at

a time following *Wöhler*'s disproof of Vitalism in 1828 and coincident with a decade when new and radical thoughts were being formulated by *Friedrich Engels* (1820–1895) and *Karl Marx* (1818–1883), who met up in Paris in 1844 and criss-crossed Europe while chasing the revolutions of the time, progress in the analysis of bile was, however, perhaps not universally accepted. Some offered a less than sanguine perspective on bile, such as that of *J. Oliver Curran*, secretary to the council of the Dublin Pathological Society in *The Dublin Quarterly Journal of Medical Science* in 1846 (*67*):

> We have made but little allusion to the chemical history of the bile, for the very simple reason, that we believe analysis of the biliary fluid has as yet thrown no light on the subject. Although bile has been carefully examined by Berzelius, Bracconot, Bizio, Bostock, Chevreul, Chevallier, Demarçay, Fourcroy, Frommhertz, Gmelin, Gugert, Henry, Kuhn, Kemp, Lychnell, Lassaigne, Liebig, Pleischl, Prout, Thenard, Theyer, Schlosser, and many others, and each has added his mite in the form of a proximate principle, or something of the kind, to increase the complexity of this puzzling fluid, none of them found in it any sulphur; yet it was recently shown by Redtenbacher, that taurine (a proximate principle obtained by Gmelin from bile, by boiling it in hydrochloric acid) contains no less than *thirty per cent. of sulphur*. This discovery completely overthrows most of the beautiful and ingenious formulæ which we find in Liebig's book and proves how much has yet to be done before analytic chemistry can pretend to form any exclusive theory of the vital processes.

But this is getting ahead of the history of bile analysis in the first half of the 19th century, and, as shall be seen for the pigments of bile, analysis was found lacking.

Berzelius, a prodigious scientist and writer, left scientific accomplishments recorded in the 27 volumes of his *Årsberätelse* (yearly reports to the Swedish Academy on the progress of chemistry and physics between 1821 and 1848, the year of his death), and in five editions comprising many volumes of his *Lehrbuch der Chemie*, the all-encompassing summaries on the same subjects published as a first edition in 1803–1818 and culminating in the fifth edition in 1843–1848. The *Årsberätelse* were translated from the original Swedish into German as *Jahres-Berichte* by *Friedrich Wöhler*. They were also translated into other languages, *e.g.* French, and *Berzelius*' *Lehrbuch* became the most comprehensive reference on chemistry in the 19th century.

Berzelius' analyses of bile covered more than 40 years from 1807. Clearly between 1812 and 1842 *Berzelius* continued his studies on bile, presenting his findings annually to the Swedish Academy of Science and leaving them to be translated into German and published variously in his *Jahres-Berichte*, which in 1828 (*68*) included a report on bile, and in his *Lehrbuch*, which in 1831 (*69*) included an update of his analysis of bile and was translated from German into French in 1833 (*70*), in 1840 (*71*), and in 1842 in *Wagner*'s *Handwörterbuch der Physiologie* (*72*). His subsequent long reports in 1840 and 1842 in the early research journals, *e.g. Annalen der Chemie und Pharmacie* (*73*, *74*) (*Liebig's Annalen*), and more concise versions in 1840 and 1842 in the *Journal für praktische Chemie* (*75*, *76*) focus exclusively on bile. These works and others, albeit repetitious, summarize *Berzelius'* nearly four decades of research on bile, which was, of course, only a small fragment of his much vaster and doubtless more earth-shaking contributions to science (*22*, *77*).

Shortly after the 1826 report on bile and gallstones by *Tiedemann* and *Gmelin* (*48*), in his *Jahres-Bericht* of 1827 ("Ueber die Fortschritte der physischen Wissenschaft"), *Berzelius* summarized his studies of ox, dog, and human bile, from which he separated a number of components (*68*): (1) a musk-like odorous/malodorous material from ox bile that co-distilled with water; and (2) from ox bile dried by gentle warming: *Gallenfett* (*Cholesterin* – cholesterol), *Oelsäure* (oleic acid) and *Margarinsäure* (margaric or heptadecanoic acid) which he obtained collectively by extraction into alcohol, then separated by various manipulations; (3) *Gallenharz*, softer at room temperature than wax but firmer than turpentine and of a dark green-brown color, obtained from the lead sulfate precipitates obtained during the isolation of *Margarinsäure*; (4) *Gallensäure* (*Acidum cholicum*, *Cholsäure*) or cholic acid, named to avoid confusion with *Gallasäure*, which was discovered in bile by one of the authors ("…ist eine von den Verfassern in der Galle entdeckte, vorher unbekannt gewesene, Säure"), a previously unknown acid and which contains nitrogen, released NH_3 in dry distillation – doubtless it was not pure cholic acid, and clearly both carbon-hydrogen and nitrogen combustion analysis were being used along with the obsolescent dry distillation; (5) *Gallenspäragin* ("wie die Verfasser auch selbst zugeben"), also claimed to be discovered by *Berzelius*, obtained as a consequence of the various manipulations above that yielded the *Gallenharz*, mixed with asparagine and *Gallenzucker* and separated; (6) *Gallenzucker* (*Thenard*'s *picromel*) obtained from *Gallenharz* by manipulations and precipitation involving treatment with basified lead oxide, eventually yielding a bright yellowish mass of irregular granular crystals; (7) *Farbstoff*, the coloring matter of bile; (8) *Gliadin*, obtained during the separation of *Gallenharz*; (9) *Schleim* (mucus) from the gallbladder, obtained by heating in water the substance remaining after dried bile is treated with alcohol; (10) *Käsestoff* (cheesy material) mixed with *Speichelstoff* (ptyalin), the water-insoluble substance obtained after drying the decoction above and heating the mass in alcohol; (11) a unique nitrogen-containing, yellow-colored substance that is soluble in water and insoluble in alcohol; (12) *Fleischextract* (meat extract) *Osmazom*, which remained behind with the *Gallenzucker* precipitated by vinegar of lead (a solution of basic lead acetate); (13) a substance with a urine-like odor obtained after calcining or heating red hot; (14) Na_2CO_3 and $(NH_4)_2CO_3$; (15) sodium acetate; (16) sodium and potassium oleate, margarate, bile acid salts, sulfates and phosphates, and NaCl, calcium phosphate, and 91.51% water.

The last was especially telling and reinforced what others concluded: bile is >90% water. In 1827, *Berzelius* doubted, however, that all of the materials that he isolated are actually present intact in bile, and he strongly suspected, having investigated the chemical composition of bile 20 years earlier, that many of the separated components were in fact artifacts of the isolation processes, *i.e*. the original components of bile had suffered transformations – a concept disputed at the time by *Chevreul* as well as *Gmelin*. In fact, *Berzelius* believed even 20 years earlier that bile actually had a simpler composition than that summarized above (*68*):

> Es entsteht hierbei nun die Frage: Finden sich alle diese Stoffe in der Galle, oder sind sie durch die Einwirkung der Reagentien auf einen oder einige Bestandtheile der Galle, deren

Zusammensetzung leicht verändert wird, erzeugt worden? Als ich vor 20 Jahren die chemischen Verhältnisse einiger thierischen Stoffe untersuchte, glaubte ich zu finden, dass sie durch gewisse Reagentien Veränderungen erlitten und neue Producte entstünden, und ich hielt insbesondere Kochen mit Wasser, Aether oder Alkohol für weniger anwendbar, da die beiden letzteren aus Eiweiss, Faserstoff, Leim u. a. ein Fett von einem eigenen widrigen Geruch hervorbrachten (vergl. Jahresb. 1826, p. 277.). Diese Ideen sind von Chevreul bestritten worden, und Leopold Gmelin hält hierbei Chevreul's Ansicht für die richtigere…

Bekanntlich wird die von dem Gallenblasenschleim befreite Galle durch Säuren, und vorzüglich durch Schwefelsäure, auf die Art zersetzt, dass die Säure, bei einer gewissen Concentration, eine harzartige Substanz ausfällt, die etwas in Wasser und vollkommen in Alkohol auflöslich ist. Dabei bleiben in der sauren Flüssigkeit nur Fleischextract und Salze zurück. Bei einer Analyse, die ich vor mehr als 20 Jahren mit der Galle auf diese Weise anstellte, glaubte ich zu finden, die Galle habe eine ganz einfache Zusammensetzung, es seien nämlich die eiweissartigen Bestandtheile des Blutes in eine eigene Substanz verwandelt worden, die, wie jene, die Eigenschaft hätte, von Mineralsäuren, nicht aber von Essigsäure, gefällt zu werden; wobei die Flüssigkeit, worin diese Substanz aufgelöst war, fast von gleicher Natur mit der wäre, worin das Eiweiss und der Faserstoff im Blute aufgelöst sind. Aus der Verbindung mit Schwefelsäure konnte diese Substanz durch Digestion mit kohlensaurem Baryt wieder erhalten werden, wobei sie mit ihren vorigen Eigenschaften wieder im Wasser auflöslich worde. Ich nannte sie Gallenstoff. – Diese Versuche sind von Gmelin und Tiedemann wiederholt worden; sie fanden dabei, dass die Schwefelsäure den Gallenstoff ausfällte, dass aber die, durch Digestion mit kohlensaurem Baryt erhaltene Auflösung davon barythaltig war, und dass in dem im Ueberschuss angewandten kohlensauren Baryt Gallenharz unaufgelöst zuruckblieb. Sie schlossen daraus, dass Schwefelsäure Essigsäure mit dem Harze gefällt habe (ein gewiss ganz ungegründeter Schluss), dass diese Essigsäure Baryt aufgelöst habe, und dass die von mir Gallenstoff genannte Substanz eine Zusammensetzung aus Gallenharz, Farbstoff, Gallenzucker, Asparagin, Gallenfett, Margarinsäure, Oelsäure etc. und Esigsaurem Baryt sei. Dieser Schluss kann nicht richtig sein, denn wenn auch die Zusammensetzung der Galle nicht so einfach ist, wie aus meinen Versuchen hervorgehen würde, so lässt sich doch mit Gewissheit sagen, dass sich nicht 7 verschiedene organische Stoffe mit einander vereinigen, um einen einzigen Stoff von so bestimmten Characteren hervorzubringen, wie der ist, mit Schwefelsäure und anderen Mineralsäuren Harz zu bilden und von Essigsäure nicht gefällt zu werden; und wie sollten Oelsäure und Margarinsäure in eine solche Verbindung mitfolgen, da doch ihre Verbindung mit Baryterde unauflöslich ist. Was den Barytgehalt betrifft, so ist diese Beobachtung richtig; nicht allein Baryterde, sondern auch Kalkerde und Bleioxyd * [*) Lychnell hat einige Versuche angestellt, um die durch ungleiche Behandlung der Galle entstehende Verschiedenheit im analytischen Resultate auszumitteln. Bei einem dieser Versuche wurde schwefelsaurer Gallenstoff in Alkohol aufgelöst und mit kohlensaurem Baryt digerirt, bis die Flüssigkeit neutral wurde. Beim Abdampfen hinterliess die Auflösung eine in Wasser vollkommen auflösliche, der Galle ähnliche Substanz, die aber beim Verbrennen kohlensauren Baryt hinterliess. Dasselbe fand mit kohlensaurem Blei statt, aber die Auflösung wurde nicht neutral. Beim Verdünnen mit Wasser fiel schwefelsaurer Gallenstoff nieder, und nach dem Filtriren und Abdampfen blieb dieselbe Substanz, wie vorher, zurück, entheilt aber nun Bleioxyd. Als zu der Auflösung der sauren Verbindung in Alkohol kohlensaures Kali gesetzt wurde, entstand schwefelsaures Kali und eine regenerirte Galle. Ich hoffe, künftig die Resultate von Lychnell's Versuchen ausführlicher mittheilen zu können.] womit man die Schwefelsäure wegnimmt, verbinden sich mit der Substanz, die jene verlässt, und löst sich damit in Wasser auf, wenn nicht die Digestion mit einem Ueberschuss der Base zu lange fortgesetzt wird, wodurch sich eine unauflösliche Verbindung bildet, und es ist hier keine Säure, sondern der thierische Stoff, der die Base auflöst. Er hat in diesem Falle mit mehreren anderen organischen Stoffen Aehnlichkeit, vor allen aber besonders mit

dem Süssholzzucker, der mit der Schwefelsäure und den Säuren im Allgemeinen harzähnliche Verbindungen bildet, und der bei ihrer Zersetzung mit einer kohlensauren Basis, z. B. kohlensaurem Baryt, Baryterde aufnimmt und damit in Wasser auflöslich wird.Legt man noch die zwischen Gallenstoff und Süssholzzucker bestehende Aehnlichkeit im Geschmack zusammen, so wird die Uebereinstimmung noch auffallender.

Wäre Asparagin in der Galle aufgelöst enthalten, so würde diese Substanz mit dem Schleim unaufgelöst zurückbleiben, wenn eingetrocknete Galle in Alkohol aufgelöst wird; diess geschieht gleichwohl nicht, und Gmelin und Tiedemann bemerken, dass es nicht einmal der Fall sei, wenn die mit Essigsäure versetzte und zur Trockne abgedampfte Galle mit Alkohol behandelt wird, wobei doch die Affinitäten der Säure das Band aufgelöst haben müssten, wovon man glauben könnte, dass es diese Substanzen in Verbindung halte. Es geht hierans ziemlich gewiss hervor, dass sich das Asparagin nicht in der Galle vor der Einwirkung gewisser Reagentien befindet; aber zu gleicher Zeit, wenn Asparagin aus irgen einem Bestandtheil der Galle entsteht, müssen auch andere Stoffe gebildet werden, und könnten in Folge hiervon nicht zuvor in der Galle enthalten gewesen sein.

Hierbei ist indessen zu bemerken, dass wenn auch die Zusammensetzung der Galle einfacher wäre, als es aus den vorhergehenden Versuchen scheinen würde, es doch nicht zu bestreiten ist, dass das Interessanteste unserer Kenntniss von der Galle die Bekanntschaft mit den vorzüglichsten Veränderungen ist, die sie durch Reagentien ausserhalb des Körpers erleidet, wodurch wir einen Theil der Veränderungen voraussehen können, die wie in dem lebenden Körper beim Digestionsprozess erleidet. 13

Despite the curious and interesting results in *Berzelius'* 1827 report (*68*), for our purposes item (7) above, *Farbstoff*, is the most relevant. *Berzelius* indicated that, "as everyone knows" (*bekanntlich*), bile from the human gallbladder was yellow, which he said was the result of a characteristic pigment component of bile. He noted then that no method for its extraction from bile had as yet been discovered but reassured that its existence had nevertheless been proven. He cited *Thenard*'s work in which he found that the yellow pigment was the main component of ox gallstones, which formed a brownish-yellow, easily-pulverized mass, from which when a little crystalline fat was removed by heating in water, caustic ammonia (NH_4OH) dissolved the yellow pigment. The latter changed color to grass-green in air and became pale red with HNO_3 and lost its color with Cl_2. It was found to be dissolved best in aq. potash to form a yellow-brown solution that gradually turned green. Addition of hydrochloric acid to the green solution yielded an emerald green precipitate that dissolved in caustic ammonia, and also in HNO_3 with a rose-red color that gradually went over to yellow. *Berzelius* noted that bile behaved in the same way and indicated that dog bile when protected from air and mixed with hydrochloric acid did not go green. The very same result was found by *Tiedemann* and *Gmelin* (*48*). How much of *Berzelius*' report recapitulated the latter's work is unclear, but what he made clear was that the thusly protected bile went green when air was absorbed, and that all acid-treated bile went green upon evaporation in air. Moreover, that every sort of bile, mixed in small portions with HNO_3 undersent the color changes of the *Gmelin* reaction: the yellow bile changing first to green, then blue, violet and next to red – all in the course of a few seconds before finally turning to yellow. *Berzelius* noted further that when the green stage of the *Gmelin* reaction was quenched with excess KOH, the liquid became brownish-yellow, and at the blue or violet stage it became yellow-green. Addition of H_2SO_4 restored the first color, *etc.* (*68*):

VII. Farbstoff. Bekanntlich färbt die Galle alle die Gallenblase umgebenden Theile gelb, Leberkranke bekommen von der absorbirten Galle eine gelbe Farbe etc., und diess rührt von einem in der Galle entkräftenen eigenen Farbstoff her, zu dessen Ausziehung sie gleichwohl keine Methode ausfindig machen konnten, dessen Existenz aber doch dargethan werden kann. Thénard glaubte gefunden zu haben dass dieser Farbstoff die Hauptmasse der bei den Ochsen so gewöhnlichen Gallensteine ausmache. So wie er darin vorkommt, bildet er eine braungelbe, leicht pulverisirbare Masse. Kochendes Wasser sich daraus ein wenig, nicht krystallinisches Fett aus und färbt sich blassgelb. Kaustisches Ammoniak nimmt mehr davon auf die Flüssigkeit ist gelb, färbt sich an der Luft grasgrün wird von Salpetersäure blassroth und verliert durch Chlor die Farbe. Am besten löst er sich in Kali auf; die Auflösung ist gelbbraun und wird allmählig grünlich. Salzsäure fällt dann diese Auflösung mit grüner Farbe. Der Niederschlag wird von Salzsäure mit smaragdgrüner, von Salpetersäure mit rosenrother Farbe aufgelöst, die allmählig in eine gelbe übergeht. Der grüne Niederschlag mit Salzsäure wird leicht von kautischem Ammoniak aufgelöst. * [*Dieselben Verhältnisse sind von Lassaigne und Leuret hei dem gelben Farbstoff in der Haut und den Flüssigkeiten von Kindern bemerkt worden, die mit Gelbsuch geboren waren. Journ. de Ch. med. II, p. 264.] – Diese Verhältnisse zeigt auch die Galle. Vermischt man Hundegalle in einer umgestülpten Glasröhre über Quecksilber mit Salzsäure, so venändert sich die Farbe nicht, lässt man aber Sauerstoffgas zu, so wird eine Portion davon absorbirt und die Flüssigkeit färbt sich grün. Auf gleiche Weise wird alle mit Säure versetzte Galle beim Abdampfen in der Luft grün. Jede Art von Galle, in kleinen Antheilen mit Salpetersäure vermischt, färbt sich zuerst grün, dann blau, violett, und darauf roth; und zwar nach einigen Secunden; nach längerer Zeit oder durch mehr Säure wird sie zuletzt gelb. Durch diese Reaction kann die Gegenwart von Galle bei Krankheiten im Serum und im Urin entdeckt werden. Wird eine mit Salpetersäure grün gefärbt Hundegalle mit Kali gesättigt, so wird sie braungelb, in's Grünliche; war sie blau oder violett, so wird die alkalische Flüssigkeit gelbgrün. Zugesetzte Schwefelsäure bringt wieden die erste Farbe hervor. Sättigt man eine mit Salpetersäure blaugefärbte Galle mit Kalk und setzt hierzu, ohne umzurühren, concentrirte Schwefelsäure, so hat man über der Säure, die zu Boden gesunken ist, Schichten von verschiedenen Farben, nämlich der Säure zunächst roth, dann blau, dann grün und zuletzt gelbgrün. 14

Soon thereafter, in 1831, *Berzelius* published an update on his isolations from bile in the second edition of his *Lehrbuch*, volume 4 (*69*, *70*). He described the color of bile as green, from yellowish-green to emerald green, and the substance as bitter tasting and of a peculiar nauseating odor; then he recounted his 1827 description of the pigment of bile, with some modification. He associated the yellow color of bile with the yellow color of jaundice that is seen in the skin and the eyes, with its source being the gallbladder. He indicated further that *Thenard* found it precipitated in human bile as a yellow powder, which he named *la matière jaune de la bile* and showed that it is the same as the yellow substance found in ox gallstones and is also the same as the yellow pigment found in the bile duct of a dead elephant of *le Jardin du Roi*, Paris – with an accumulated weight/mass amounting to 1.5 pounds (*61*). *Berzelius* wrote further that, led by *Gmelin's* investigation of ox gallstones and assertion that the yellow pigment comprises their main component, he then ground gallstones into a red-brown powder, which he heated in alcohol (to remove only a little fat) and found that caustic ammonia (NH_4OH) dissolved only a little of it but that caustic potash (KOH) was more effective and became brightly yellow colored but turned green-brown due to absorption of O_2 from air. When strongly saturated with HNO_3, within in a few seconds it displayed the color changes of the *Gmelin*

reaction, as is characteristic of bile. He further indicated that the *Gmelin* reaction was the most certain means for detecting the presence of bile or its pigment. With added hydrochloric acid, the KOH solution formed a precipitate of dark green flakes, leaving a solution with a tinge of green. After washing and drying, the green precipitate was soluble in HNO_3, in which it raised a red color without blue or violet in between that quickly turned yellow. Characteristically, as indicated in 1826 by *Gmelin* (*48*), the yellow color of bile underwent the *Gmelin* reaction with HNO_3, and the yellow color change from yellow to green in bile occurred by oxidation from oxygen in the air – but remained yellow in the absence of air. *Berzelius* thus concluded that the green color encountered in bile originates from the yellow pigment – by oxidation, and that the green pigment was more soluble in alkali, which rendered difficult the separation of the two commingled pigments.

In 1831, *Berzelius* wrote (*69*):

> 9) *Farbstoff*. Die grünliche Farbe der Ochsengalle gehört, aller Wahrscheinlichkeit nach, einer eigenen Substanz an, die mit den übrigen Stoffen in der Galle aufgelöst ist, und die sich zwar auf analytischem Wege bisher noch nicht mit Zuverlässigkeit abscheiden liess, sich aber bei krankhaftem Zustande in der Galle bisweilen in so grosser Menge absetzt, dass sie eine eigene Art Gallensteine bildet, durch die man sie eben in isolirter Gestalt, hinsichtlich ihrer characteristischen Reactionen, kennen lernnen konnte. Es ist derselbe Stoff, welcher in der Gelbsucht einen grossen Theil des Körpers, wie namentlich die Haut, das Weisse im Auge u. a., gelb färbt und die Ursache der gelben Farbe ist, welche man der Gallenblase und den sie umgebenden Theilen nach dem Tode gefunden hat. Thénard machte zuerst aufmerksam darauf; er fand ihn in der Menschengalle in Gestalt eines gelben Pulvers augeschlämmt, welches er *matière jaune de la bile* nannte, und von dem er zeigte, dass sie dieselbe Substanz sei, welche man in den Gallensteinen von Ochsen finde, und auch bei einem im *le Jardin du Roi* zu Paris verstorbenen Elephanten gefunden habe, bei dem sie eine in dem Lebergallengang angesammelte Masse von 1½ Pfund Gewicht ausmachte.
>
> Zur Darlegung der Beschaffenheit dieser Substanz werde ich Gmelin's Untersuchung Ochsen-Gallensteins anführen, wovon sie den Hauptbestandtheil ausmachte, Er liess sich leicht zu einem hell rothbraunen Pulver reiben. Kochender Alkohol zog daraus nur wenig Fett aus, und färbte sich gelb. Kaustisches Ammoniak löste eine geringe Menge davon auf; das beste Lösungsmittel dafür war aber Kalihydrat. Die durch Digestion erhaltene Auflösung war hellgelb, und wurde durch Sauerstoff-Absorption aus der Luft grünlichbraun. Mit Salpetersäure stark übersättigt, zeigt diese Auflösung eine Reaction, die für den Farbstoff der Galle characteristisch ist; setzt man nicht zu viel Säure auf einmal hinzu, indem man wohl ummischt, so wird die Flüssigkeit zuerts grün, darauf blau, violett und zuletzt roth, und diese Farbenveränderung geht innerhalb weniger Secunden vor sich. Nach einer Weile verschwindet auch die rothe Farbe, die Flüssigkeit wird gelb, und die Eigenschaften des Farbstoffs haben sich nun gänzlich verändert. Es bedarf nur einer sehr geringen Menge Farbstoff, um diese Reaction deutlich merkbar zu machen, und sie findet nicht allein mit Galle, sondern auch mit Blutwasser, Chylus-Serum, Urin und anderen Flüssigkeiten statt, wenn sie bei der Gelbsucht eine gelbe Farbe angenommen haben, und ist daher das sicherste Entdeckungsmittel für die Gegenwart von Galle oder ihres Farbstoffs. Die Auflösung des Farbstoffs in Kali wird von Chlorwasserstoffsäure in dicken dunkelgrünen Flocken gefällt, und nachher zeigt die Flüssigkeit nur einen schwachen Stich in's Grüne. Der niedergeschlagene Farbstoff löst sich, nach dem Auswaschen und Trocknen, in Salpetersäure mit rother Farbe, ohne blau oder violett dazwischen, auf, und die rothe Farbe geht bald in die gelbe über. Der durch Salzsäure bewirkte dunkelgrüne Niederschlag löst sich sehr leicht und mit grasgrüner Farbe, sowohl in Ammoniak als Kali auf.

> Die Ursache der in der Galle oft vorgehenden Farbenveränderungen von Gelb in Braun und Grün, scheinen auf der Oxydation des Farbstoffs zu behruhen, wobei er von Gelb in Grün übergeht, und dadurch in Alkali leichter löslich wird. Galle, mit einer Säure versetzt und Berührung mit der Luft gelassen, wird nach einigen Tagen völlig grün. Gmelin vermischte Hundegalle, die gelbbraun ist, mit Salzsäuer in einer, an einem Ende zugeschmolzenen und über Quecksilber umgestürzten, Glasröhre. Auf diese Weise vor dem Luftzutritte geschützt, blieb die Farbe des Gemisches unverändert; sowie aber Sauerstoffgas hinzugelassen wurde, färbte es sich grün, zuerst an der Berührungsfläche mit dem Gase und nachher durch und durch, indem die Galle dabei ihr halbes Volum Sauerstoffgas absorbirte. Chlor bringt dasselbe Farbenspiel wie Salpetersäure hervor, jedoch weniger lebhaft; das Blau ist kaum merklich, sondern die Farbe geht gleich von Grün in Roth über, und ein Ueberschuss von Chlor zerstört die Farbe der Galle gänzlich und bleicht dieselbe unter Bildung einer weissen Trübung. 15

All of the above was also summarized in 1834 by *Dulk* in his *Lehrbuch* (*78*), for use in his lectures and for self study, which paints a somewhat different picture from that of *Curran* (*67*) but perhaps did little to improve the latter's view. Nonetheless, bile clearly became known and described as a complicated mixture, a nearly intractable biological fluid from the viewpoint of some. Undeterred, *Berzelius* persisted in his analyses of bile into the early 1840s, motivated by scientific curiosity, the new discoveries of *Demarçay* (*63*), *Chevreul* (*44*, *45*), and others, the need to update his *Lehrbuch* with new editions (4th ed., 1835–1842; 5th ed., 1843–1848), and perhaps to re-establish his own studies and perspectives. His collection of writings published between 1840 and 1842 may well have expressed his then most recent and final thoughts on the components of bile. These works, while oriented toward the newly-discovered major (lipid) components of bile, especially the bile acids, fatty acids and their salts did address the yellow and green pigments of bile.

In the 3rd edition, 9th volume of his *Lehrbuch*, published in 1840 (*71*), *Berzelius* reviewed the early work and progress since 1807, his own, *Fourcroy*'s, *Thenard*'s, *Gmelin*'s, as well as the then more recent work of *Demarçay*, *Frommherz*, and *Gugert*. In this comprehensive volume, which as in all the *Lehrbuch* was far broader than the subject of bile alone, *Berzelius* critiqued the work of other investigators while reconciling or repudiating it relative to his latest studies, which were presented in considerable detail. He wrote that the prevailing early view was that bile consisted mainly of *Gallenharz* and picromel (*71*):

> Diese Ansicht wurde hierauf die herrschende, und alle später angestellten Analysen gingen von der Idee aus, dass die Galle hauptsächlich aus Picromel und Gallenharz bestehe. 16

Berzelius' approach in 1840 (*71*) to initiating the separation of bile into its components seemed twofold: (1) first adding H_2SO_4, followed by manipulations involving barium salts, or (2) adding lead salts (*71*):

> Wie erwähnt wurde, kann die Analyse der Galle auf zweierlei Art geschehen, nämlich durch Schwefelsäure oder durch Bleisalze; allein sie muss, damit so viel wie möglich Metamorphosen vermieden werden, mit andern, als den bis jetzt angewandten Vorsichtsmaasregeln angestellt werden. 17

(1) Analysis of bile using H_2SO_4. First ox-bile was evaporated over H_2SO_4 between 100° and 110°, taken to dryness in order to be pulverized. The powder

was digested 2–3 times with dry ether in order to remove fats, and the digested powder was taken up in anhydrous alchohol to leave behind mucus (*Schleim*), NaCl, and other alcohol-insoluble salts and animal substance but dissolving a compound of the bitter component of bile with alkali, alkali oleate, and margarinate, the pigments of bile in a similar compound, *etc*. The solution obtained was filtered and the residue was washed with anhydrous alcohol. The residue was washed with 85% alcohol, which dissolved certain substances, and then retained. The anhydrous alcohol solution above was then mixed in small portions, with shaking, with a solution of $BaCl_2$ in H_2O until a dark green precipitate had formed. The green precipitate was filtered and washed with alcohol, which however was not required to be anhydrous. Baryta water (aq. BaO) was added dropwise to the filtered solution. The precipitate thus formed was first dark gray colored but became green after a few moments. Baryta water was added as long as the solution was still cloudy. The precipitate was soon no longer green, but only yellow-brown, and finally only yellow, whereupon the solution had for the most part lost its color, and showed only yellow in it. The precipitate was filtered and washed with 84% alcohol (*71*):

> 1. Analyse der Galle durch Schwefelsäure – Die Ochsengalle wird im Wasserbade oder im leeren Raum über Schwefelsäure verdunstet, indem zuletzt die Temperatur in dem leeren Raum auf + 100° bis +110° steigen muss, damit die Masse so trocken wird, dass sie zu Pulver gerieben werden kann. Dann wird sie mit wasserfreiem Aether übergossen. Ist der Aether wasserhaltig, so nimmt die Galle das Wasser auf und fliesst zusammen. Der Aether zieht alles Fett aus, welches nicht mit Alkali zu Seife verbunden ist. Das mit Aether zwei bis drei Mal digerirte Pulver wird darauf in wasserfreiem Alkohol aufgelöst, welcher Schleim, Kochsalz und andere in Alkohol unlösliche Salze und Thierstoffe zurücklässt, dagegen eine Verbindung des bittern Bestandtheils der Galle mit Alkali, ölsaures und margarinsaures Alkali, den Farbstoff der Galle in einer ähnlichen Verbindung, u. s. w., auflöst. Die erhaltene Lösung wird filtrirt und das Ungelöste zuerst mit wasserfreiem Alkohol gewaschen, der dann der filtrirten Lösung zugefügt wird, und darauf mit Alkohol von 0,85, welcher gewisse Stoffe daraus auflöst, und der für sich genommen wird. Die Lösung in wasserfreiem Alkohol wird nun in kleinen Portionen und unter Umschütteln mit einer Lösung von Chlorbarium in Wasser vermischt, so lange noch ein dunkelgrüner Niederschlag gebildet wird, den man abfiltrirt und mit Alkohol, der jedoch nicht wasserfrei zu sein braucht, abwäscht. Zu die filtrirten Lösung tropft man dann Barytwasser. Der Niederschlag, welcher dadurch gebildet wird, ist anfänglich dunkelgrau, färbt sich aber nach einigen Augenblicken grün. Das Barytwasser wird so lange zugesetzt, als die Lösung noch dadurch getrübt wird. Der Niederschlag wird bald nicht mehr grün, sondern erst braungelb, und zuletzt nur gelblich, worauf die Lösung ihre Farbe grösstentheils verloren hat, und sich nur noch in's Gelbe zieht. Der Niederschlag wird abfiltrirt, und mit Alkohol von 0,84 ausgewaschen. 18

The residual ethanolic solutions from approach (1) that contained free BaO/$Ba(OH)_2$ was precipitated as $BaCO_3$ with CO_2 gas, filtered, and evaporated to dryness before processing further with PbO, *etc*. to yield *Bilin* (named by *Berzelius* from *Bilis*, bile) that is identical to *Gmelin*'s *Gallenzucker*, a procedure similar to that which led *Thenard* to isolate a component that he called *Picromel* ($\pi\iota\kappa\rho o\zeta$, bitter, and $\mu\varepsilon\lambda\iota$, honey). Isolated as the metamorphosis products of *Bilin* were (as named by *Berzelius*): *Fellinsäure* (from *Fel fellis*, bile), *Acidum fellicum*, *Cholinsäure* (from $\chi o\lambda\eta$, bile), *Acidum cholonicium*, and *Dyslysin* (from $\delta\upsilon\zeta$, difficult, and $\lambda\nu\delta\iota\zeta$, solution).

(2) Analysis of bile from lead salts. In this approach, dilute acetic acid is added to fresh gallbladder bile to separate mucus (*Schleim*), mixed with twice the volume of alcohol then processed further using PbO to yield bile acids and their salts, *inter alia*, bile acids akin to those isolated by *Demarçay*. Thus, as described in his 1840 *Lehrbuch* (volume 9) (*71*), *Berzelius* found that approaches (1) and (2) could be used to precipitate biliverdin (as its barium salt) and *Bilifulvin* (bilifulvin), also as its barium salt, but mainly (1) and (2) served as the entry point to separate out the many other more major components of bile. For *Gmelin* in ~1826 (*48*) it led to previously unknown components such as taurine and cholic acid as well as a substance he named (bittersweet) *Gallenzucker*, and much more (*71*):

> Gmelin fand ausser diesen Bestandtheilen, nämlich dem Gallenharz und Gallenzucker, noch Taurin, Cholsäure, Cholesterin, Oelsäure, Margarinsäure, Farbstoff, Fleischextract, eine extractähnliche unrinöse Substanz, eine dem Pflanzenleim analoge Materie, Käsestoff, Speichelstoff, Albumin, Schleim, kohlensaures Natron, kohlensaures Ammoniak, essigsaures (milchsaures) Natron, ölsaures, margarinsaures, cholsaures, schwefelsaures und phosphorsaures Natron und Kali, Kochsalz und phosphorsauren Kalk. 19

And the discussion led to *Berzelius*' new term, *Bilin*, which he said was identical to *Gallenzucker* and numerous other lipid and inorganic products. For *Demarçay*, it opened the door to bile acids and their salts and created quite a stir with *Berzelius*, who was motivated to devote numerous pages of his *Lehrbuch* to further explanations of the lipid components (*71*):

> Demarçay leugnet gänzlich die Existenz eines Gallenzuckers und hält Gmelin's Gallenzucker und Thénard's Picromel für identisch mit *Acide choleique*.
>
> Nachdem wir auf diese Weise innerhalb eines Zeitraums von mehr als 30 Jahren hinsichtlich des Hauptbegriffs von der Natur der Galle in einem Zirkel gegangen sind, freilich nicht ohne bedeutende Vermehrung unserer Kenntnisse, stehen wir wieder auf demselben Punct, und ungeachtet aller der Erfahrunge, die wir durch die angeführten Arbeiten gewonnen haben, wäre es doch nicht möglich ohne neue Untersuchungen einen nur einigermaassen richtigen Begriff von der Zusammensetzung der Galle zu geben. Ich werde sie nun abhandeln nach den Untersuchungen, die ich neuerlich in dieser Absicht mit der Ochsengalle angestellt habe. 20

Though *Berzelius'* discussion of the pigments of bile and their separation was much less extensive than that of other components of bile, he described the isolation of the two pigments of bile, initiated by approach (1) and followed by several manipulations before precipitation with $BaCl_2$. Two different barium precipitates were obtained, the first from addition of $BaCl_2$, followed by a second addition of BaO or $Ba(OH)_2$, and each yielded a different pigment. The first, which gave bile its green color was bound to baryta, *Berzelius* named *Biliverdin* (coming from *bilis*, bile, and *verdire*, green). The second, brownish precipitate formed from baryta water and contained, beside biliverdin, a reddish-yellow (orange) pigment that *Berzelius* named *Bilifulvin* (from *Bilis*, bile, and *fulvus*, reddish-yellow), an extractable substance and characteristic nitrogen-containing animal substance which *Berzelius* would return to later.

These pigments were doubtless mixtures of barium salts (*71*):

> Der erste Niederschlag mit Chlorbarium enthält den Stoff, welcher der Galle ihre grüne Farbe gibt, verbunden mit Baryterde. Ich nenne ihn *Biliverdin* (von Bilis, Galle, und verdire,

grün werden). Der andere, oder der Niederschlag mit Barytwasser, enthält neben dem Biliverdin einen rothgelben Farbstoff, welchen ich *Bilifulvin* (von Bilis, Galle, und fulvus, rothgelb) nenne, einen extractähnlichen Stoff und einen eigenthümlichen, stickstoffhaltigen Thierstoff, auf welche ich weiter unte zurückkommen werde. 21

Possibly a better way to access the green pigment was found in yet a third approach, where dried bile, dissolved in alcohol, was treated with $BaCl_2$ – a method similar to (1) but still employing $BaCl_2$ to precipitate the green pigment. The precipitate was digested with hydrochloric acid to remove BaO, washed with ether to remove fat, and processed with cold anhydrous alcohol to yield a green-brown solution and a green insoluble residue. The alcohol solution, allowed to evaporate on its own, yielded biliverdin in the form of a nearly black-brown, earthy compound. When evaporated with heating, it formed a shiny, translucent dark-green film (*71*):

6. *Biliverdin*, Gallengrün. Der Niederschlag wird noch feucht mit verdünnter Salzsäure übergossen, welche die Baryterde auszieht und Biliverdin zurücklässt. Er ist nur mit wenig Fett vermischt, welches man mit Aether auszieht, in dem sich jedoch auch ein kleiner Theil von dem Biliverdin gleichzeitig auflöst. Das Zurückbleibende wird mit kaltem wasserfreien Alkohol behandelt, welcher sich davon grünbraun färbt, der aber einen grünen, in kaltem Alkohol, unlöslichen Rückstand zurücklässt. Die Lösung in Alkohol, der freiwilligen Verdauung überlassen, lässt das Biliverdin in Gestalt eines fast schwarzbraunen, erdigen Körpers zurück. In der Wärme verdunstet, bildet es einen glänzenden, durchscheinenden, dunkelgrünen Ueberzug. 22

After providing a long list of the properties of biliverdin, *Berzelius* wrote that those properties corresponded altogether with all "three modifications" (*79*) of chlorophyll. He indicated that his assessment was valid not only for biliverdin from ox bile but might extend to bile from other herbivores. He said further that biliverdin from the bile of carnivores possessed quite different properties (or was tied up with a pigment not yet separated), on which he himself had not yet been able to carry out a few experiments (*71*):

Diese Eigenschaften des Biliverdins stimmen in Allem mit denen des Chlorophylls überein, so dass ich entschieden bin, dasselbe als damit identisch zu betrachten, und ich habe es aus verschiedenen Gallen in allen drei Modificationen des Chlorophylls erhalten. Das jetzt Angeführte gilt natürlicherweise nur für das Biliverdin aus Ochsengalle, vielleicht auch für das aus der Galle anderer grasfressender Thiere. Aber in der Galle fleischfressender Thiere besitzt es ganz andere Eigenschaften, oder es ist darin mit noch einem anderen Farbstoff verknüpft, von dem man es bis jetzt noch nicht geschieden hat. Da ich noch nicht Gelegenheit hatte, darüber selbst einige Versuche anzustellen, so muss ich nach Angaben Anderer berichten. 23

Berzelius' correlation of biliverdin to chlorophyll (and to the bile of "grass eaters") is rather startling and apparently did not come from a lack of experience with chlorophyll because simultaneous with his work on the pigments of bile, *Berzelius*, whose experimental work ranged far and wide in chemistry, had also been working on the isolation and properties of the green pigment of leaves in the 1830s (*79*). Though chemical studies of the green pigment of leaves dates back to the 1780s (*80*), the name *chlorophyll* was coined for green colorant of plants (after the Greek words for "leaf" and "green") by two apothecaries in Paris, *Pierre-Joseph Pelletier* (1788–1842) and *Jean Bienaimé Caventou* (1795–1877), who taught at the École de Pharmacie in 1817 (*81*).

Reddish-yellow (orange) bilifulvin on the other hand was not present in sufficient amounts in 1840 for *Berzelius* to study and exhibited color changes during its separation from an alcohol solution: first brown, then green before turning brown again and precipitating as a brownish-yellow barium salt. There was no evidence that air or light were excluded in the preparation. The separated solution was treated with sugar of lead [lead(II) acetate] solution to give a dark gray-green precipitate and became orange. Then it was precipitated with vinegar of lead (aqueous solution of basic lead acetate), but it could not be precipitated so that the solution lost its color entirely. When the precipitate had sunk to the bottom, it showed a mixture of two (compounds), of which one was reddish-yellow and heavy and lay below (the other). The upper precipitated layer, a yellowish and lighter precipitate, could not be completely separated mechanically with certainty. When it was filtered, washed, and then decomposed with H_2S, a yellow solution was obtained that left a reddish-brown extract upon evaporation. It was dissolved in alcohol and the solution was left to evaporate on its own, which led to the formation initially of reddish-yellow crystals, and then, with further evaporation, a brownish-red extract formed. The crystals were the substance that *Berzelius* named *Bilifulvin* (*71*):

> *7*. *Bilifulvin* habe ich eine noch problematische, aus Bilis bubula spissata erhaltene, krystallisirte, rothgelbe Substanz genannt, die ich noch nicht gehörig zu studieren Gelegenheit hatte. Nachdem die Alkohollösung der Galle mit Chlorbarium ausgefällt worden, gibt eingetropftes Barytwasser einen neuen Niederschlag, der im ersten Augenblick braun ist, aber seine Farbe verändert und grün wird, worauf er braun und am Ende braungelb niederfällt. Wird er nun auf ein Filtrum genommen und gewaschen, zuerst mit Alkohol und darauf mit Wasser, so löst sich in diesem ein grosser Theil, und auf dem Filtrum bleibt Biliverdin-Baryt zurück.
>
> Die durchgegangene Lösung, mit Bleizuckerlösung versetzt, gibt einen dunklen graugrünen Niederschlag und wird rothgelb. Nun wird sie mit Bleiessig gefällt, aber sie kann nicht so ausgefällt werden; dass sie ganz ihre Farbe verliert. Wenn der Niederschlag zu Boden gesunken ist, zeigt er sich aus zweien gemischt, von welchen der eine rothgelb und schwer ist, und zu unterst liegt. Oben darauf liegt ein nur gelblicher und leichterer Niederschlag, der jedoch nicht mit Sicherheit mechanisch abzuscheiden ist. Wenn sie abfiltrirt, gewaschen und darauf mit Schwefelwasserstoff zersetzt werden, so bekommt man eine gelbe Lösung, die verdunstet ein rothbraunes Extract zurücklässt. Wird dieses in Alkohol aufgelöst und die Lösung der freiwilligen Verdunstung überlassen, so schiessen daraus zuerst kleine rothgelbe Kyrstalle an, um welche sich dann bei fortgesetzter Verdunstung ein braunrothes Extract bildet. Diese Krystalle sind es, die ich Bilifulvin genannt habe. 24

Aside from the biliverdin-chlorophyll correlation drawn by *Berzelius*, he noted rather importantly that occasionally a yellow substance was found precipitated in bile and which he believed was responsible for producing a specific class of gallstones. *Thenard* first called attention to it much earlier and named it, descriptively, *la matière jaune de la bile*.

Much of what *Berzelius* wrote on bile, in the 1840 *Lehrbuch* (*71*), was an update of his chapters on bile expressed earlier in his 1831 *Lehrbuch*, and his 1828 *Jahres-Bericht*, and his chapter on bile in *Wagner*'s 1842 *Handwörterbuch*. But sections from these sources were also published or republished in the emerging new journals of the times (*73–76*) in part nearly verbatim, in part including updates or further

comments. In his 1842 publication in the *Annalen der Chemie und Pharmacie* (*74*) he restated his isolation of biliverdin from ox bile that was presented in detail in his 1840 *Lehrbuch* section on bile. However, to this he added his new experiments, including those with human gallstones, which were pulverized to a reddish-yellow powder – as *Gmelin* had reported. Processing the powder led to conversion to green material, which he isolated as a leaf-green pigment as well as a yellow one. The green pigment was separated by various manipulations into three green modifications and each (called *Blattgrün* = leaf green, green of leaves, or chlorophyll) showed a different behavior toward HNO_3, becoming red, which did not occur with the biliverdin from ox bile. It is not entirely clear why *Berzelius* used the word *Blattgrün* when addressing the green pigment(s) of gallstones, and *Biliverdin* when addressing that from bile, except that they came from different sources. In the 1842 *Annalen*, he used the word *Chlorophyll* and not *Blattgrün* when describing identity with biliverdin (*74*):

> Das mit Wasser aus der Salzsäure gefällte Grüne verhielt sich zu Alkohol, Aether, Salzsäure, Schwefelsäure, Essigsäure und Alkalien ganz so, wie Blattgrün der ersten Modification, und das, was nicht durch Wasser gefällt worden war, aber durch kohlensauren Kalk niederfiel, ganz so, wie das Blattgrün der zweiten Modification. Und wenn der Niederschlag mit Salzsäure aus der Lösung in Kali mit Alkohol ausgekocht wurde, so blieb eine dunkelgrünes Pulver zurück, welches Alkohol, worin es schwerlöslich war, noch grün und Salzsäure gelb färbte, und welches sich also wie Blattgrün der dritten Modification verhielt. Bei dem aus dem Gallenstein erhaltenen Grün zeigte sich jedoch der Unterschied, dass es mit Salpetersäure eine rothe Flüssigkeit bildete, was mit dem aus Ochsengalle abgeschiedenen Biliverdin nicht stattfindet. Alle drei aus dem Gallenstein erhaltenen Modificationen gaben mit Salpetersäure eine rothe Flüssigkeit. Von meinen alteren Versuchen mit dem Blattgrün hatte ich noch eine kleine Portion von dem Blattgrün der zweiten Modification übrig behalten. Ich übergoss diese mit reiner Salpetersäure, und sie bildete damit eine tiefrothe Flüssigkeit, aber nur für einen Augenblick, worauf sie Stickoxydgas entwickelte und gelb wurde. Bei dem Grün aus dem Gallenstein blieb das Rothe viel länger. 25

More likely, *Berzelius* was being careful not to equate the green pigments (*74*):

> Ich habe im Uebrigen das nicht untersucht, was Salzsäure nicht ausfällt, da ich hier nur beabsichtigte, den grünen Stoff mit Biliverdin zu vergleichen, welcher aus diesem braungelben Krankheitsproducte aus der Galle verschiedener Thiere durch Alkali unter dem Einfluss der Luft hervorgebracht wird. 26

For he concluded that the green pigment of bile and that from (processed) gallstones was the same. Very significantly, he also concluded that they came about by alteration (air oxidation) of the yellow pigment of bile, which he named *Cholepyrrhin* (*74*) and which we now know as *Bilirubin*:

> Diese Versuche zeigen, dass der gelbe Körper, aus welchem diese Art von Gallensteinen besteht, in Berührung mit Luft und unter dem Einflusse von Alkalien sowohl als auch von Säuren metamorphosirt, und dass durch diese Metamorphose Blattgrün, so zu sagen auf künstlichem Wege gebildet wird; ein neues Beispiel, welches wir mehreren bereits bekannten hinzuzufügen haben, dass solche Stoffe, welche die lebende Natur hervorbringt, sich künstlich durch die Metamorphose anderer Stoffe hervorbringen lassen. Hierdurch betrachte ich es also als dargelegt, dass Biliverdin und Blattgrün wirklich identische Körper sind, und ein Product der Metamorphose des eigentlichen Farbstoffs der Galle, der

während der Analyse metamorphosirt wird, ist. Dieser Farbstoff verdient einen eigenthümlichen Namen, man kann ihn *Cholepyrrhin* (von χολη, Galle, und πυρροζ, brandgelb) nennen. 27

Cholepyrrhin was thus found in fresh ox bile as well as in gallstones, which incorporated the pigment from the bile, and it was the source of the biliverdin obtained by working up bile. Likewise, *Berzelius* maintained that the taurine, cholic acid, *etc*. were also transformation products of bile, formed during the manipulations of their isolation (*74*):

Es bleibt noch übrig, die Natur des Cholepyrrhins in unverändertem Zustande zu bestimmen, so wie auch die Producte zu untersuchen, welche sich bei seiner Metamorphose ausser dem Biliverdin bilden. Wiewohl ich bei den oben angeführten Versuchen aus Ochsengalle nicht habe denselben gelben Körper ausziehen können, welcher in den Gallensteinen enthalten ist, so ist es doch klar, dass er ursprünglich darin enthalten ist. Denn die vom Schleim abfiltrirte frische Galle zieht sich kaum merklich ins Grüne, sondern ist gelb oder bräunlich gelb. Erst während der Verdunstung wird sie dunkler und grün, indem sich dabei Biliverdin bildet und das Cholepyrrhin metamorphosirt wird. Das Biliverdin ist also, gleichwie Taurin, Cholsäure u. s. w., ein Product der Metamorphose in der Galle. 28

Although it is not entirely clear from the publication dates whether *Berzelius*' 1842 publication (*74*) in *Annalen der Chemie und Pharmacie* or whether his 1842 publication in the *Journal für praktische Chemie* (*76*) is the more recent (submission dates are not announced in either), he referred in the latter to having published an extension of his studies on bile in 1840 (*52c*), studies that he had undertaken in order to update his older *Lehrbuch, i.e.* to complete the 1840 volume 9 of the *Lehrbuch*. In 1842 (*76*), he explained that those studies could not be completed until the publication of the *Lehrbuch*, that he later continued the research and thus obtained various (or different) new views on the subject and corrected others. Those results, he said, were published in 1841 in Sweden and in 1842 (German translation) in the 1842 *Annalen der Chemie und Pharmacie* (*74*) cited above. *Berzelius* wrote that the important new results could be found in his article "Galle", printed in 1842 in *Wagner*'s *Handwörterbuch der Physiologie* (*72*). And the main results merited publication in the *Journal für praktische Chemie* (*76*). Nowadays, these repetitive efforts in publishing might be seen as an unwarranted duplication. Nonetheless, each publication is somewhat different and, in the last cited (*76*), *Berzelius* expressed his final experiments and views on the subject of bile pigments.

Thus, the first chemical separations of the components of bile, carried out by *Berzelius* (*57–60*, *68–76*), *Thenard* (*53–56*, *61*), *Tiedemann* and *Gmelin* (*48*), *Demarçay* (*63*), *Chevreul* (*44*, *45*) during 1806–1842, were summarized in 1842–1843 by *Liebig* (*82*) and *Thomson* (*83*). These summaries show the advances in knowledge of the composition of bile since 1835 (*84*). Whether little or much, one can judge for oneself, as did *Curran* in 1846 (*67*), who saw little progress. Nonetheless, the work served as the basis to follow in 1843–1850 by *Simon*, *Platner*, *Scherer*, *Heintz*, and *Virchow* who wrote on bile, either summarizing the work of their predecessor(s), while adding a few of their own studies, or describing a relationship between the pigment of urine to that of bile.

It becomes apparent that from the 1830s onward various investigations of the components of bile were typically directed far more toward the isolation and composition of its non-pigment components from a wide variety of vertebrate species, as *Strecker's*[23] summaries and studies (*85*, *86*) at the time indicate. In addition to fatty acids, investigations were clearly dominated by the newly discovered bile acids, their composition, properties, and transformations. Though bile acids and their conjugates were clearly a major focus, the particular individuals cited above also pursued the separation and analysis of pigments found in bile, especially from humans and cattle.

E.A. Platner (*Privatdozent* in Heidelberg in 1844) made some new observations on bile and introduced the use of stannous oxide to precipitate a bright green solid, which he freed (into *Weingeist*, ethanol) from (colorless) salts using a few drops of sulfuric acid (*87*, *88*). Filtration of the green liquid and addition of water precipitated the pigment. Repeated processing freed it from fat and perhaps other material, and *Platner* ended up with the green pigment, which he described as difficultly soluble in ether, more difficult even if the ether contains alcohol, odorless, of a somewhat bitter taste, insoluble in hydrochloric acid and sulfuric acid but easily soluble in aq. potash and NH_4OH (in which the green color went over into yellow). With heating, the green faded into yellow (in unrevealed solvent). Ammonia was released upon heating it in aq. potash which, if not derived from a different compound than the bile pigment, would not be identical with chlorophyll, as *Berzelius* thought (*68–76*). According to *Platner* (*87*):

> . . . Er stellt dann eine grüne, leicht zu pulvernde, harzartige Masse dar, ist unlöslich in Wasser, aber leicht löslich in Weingeist. In Aether löst er sich schwer, und um so schwieriger, je weniger der Aether Weingeist enthält. Ist ohne Geruch und von etwas bitterlichem Geschmack. Unlöslich in Salzsäure und Schwefelsäure, aber leicht löslich in Kali und Ammoniak, wobei die grüne Farbe sich in eine gelbe umwandelt. Auch beim Erhitzen verblasst das Grüne und geht in's Gelbe über. Mit Kali erwärmt, entwickelt er Ammoniak. Wenn dieses nicht von einem anderen Körper herkömmt, so kann demnach der Gallenfarbstoff nicht identisch seyn mit dem Blattgrün, wie Berzelius meint. 29

Platner then went on to describe the isolation of bile acids and other materials in bile and ended with a few interesting remarks on the bile pigments. According to his experiments, bile in air gradually underwent the same sequence of color changes as in the *Gmelin* reaction. An ethanolic solution of bile left in air for a long time gradually went over completely from green to red, a color change that he attributed to a progressive oxidation of the pigment. Interestingly, *Platner* finished with the comment that the pigment obtained by *Berzelius* from bile using BaO contained no nitrogen, that the pigment he obtained by the method described did, and that a further examination was recommended (*87*, *88*):

> Schliesslich will ich noch einige Bemerkungen über den Farbstoff der Galle machen. Die Galle wird bekanntlich durch Säuren nach und nach grün, wenn zugleich die Luft

[23] *Adolph Strecker* was born on October 21, 1822 in Darmstadt and died on November 7, 1871 in Würzburg. He received the Dr. phil. in 1842 at Giessen, habilitated with *Liebig* at Giessen and became lecturer, then Professor at the University of Christiana in Norway in 1851, and Professor at Tübingen following *Gmelin*'s death. He moved to Würzburg in 1870.

beitreten kann. Augenblicklich is aber die Farbenveränderung der Galle durch Salpetersäure. Sie wird zuerst grün, dann blau, violett, und zuletzt gelb. Nachher zerstört die Salpetersäure den Farbstoff. Nach meinen Erfahrungen bewirkt jedoch der blosse Zutritt der Luft nach und nach ganz dieselben Farbenveränderungen. Setzt man nämlich eine weingeistige Auflösung der Galle längere Zeit der Luft aus, so wird sie zuerst grün, geht aber nach und nach in eine vollkommen rothe Färbung über. Diese Farbenveränderungen entstehen demnach ohne Zweifel durch eine fortschreitende Oxydation des Farbstoffs. Der von Berzelius aus der Galle mit Hülfe von Baryt dargestellte Farbstoff enthielt keinen Stickstoff. Der von mir auf die oben angegebene Weise dargestellte Gallenfarbstoff ist aber stickstoffhaltig. Möge daher auch dieser Gegenstand einer weiteren Prüfung empfohlen seyn. 30

Simon[24] wrote on the constituents of bile (*89*), following his report in 1840 (*90*) on urea from urine and components of the meconium (which is green) from children and feces from 6-day-old children nursed with mother's milk. He separated various components from the meconium (cholesterol, picromel, *etc*.) and *Gallengrün* (4%), and he also found *Gallenfarbstoff* (bile pigment) in the feces. But these were not further identified. In his section on the coloring matter of bile (*89*), for reasons unclear and unstated, *Simon* introduced a new name for the brownish-yellow pigment: *Biliphäin*, which *Berzelius* had named *Cholepyrrhin*. Despite the intrusion of a new name, *Simon*'s report on bile serves as a useful summary of what was known (*ca*. 1845) in its English translation of the original German by *George E. Day* of the Royal College of Physicians. Whether *Simon* was simply summarizing the results of previous workers (*Berzelius* in particular) or whether he repeated the experiment of *Berzelius* is not entirely clear; however, the latter seems probable (*91*):

II. THE BILE

a. The most important colouring matter of the bile is that to which it owes its characteristic brownish yellow tint. It is termed *cholepyrrhin* by Berzelius, and *biliphæin* by Simon. We shall adopt the latter term. On the gradual addition of nitric acid to a fluid that contains this substance in solution, a very characteristic series of tints are evolved. The fluid becomes first blue, then green, afterwards violet, and red, and ultimately assumes a yellow or yellowish brown colour.

All attempts to isolate this substance from the bile, by chemical means, have failed; it is apparently decomposed by the processes that are adopted in the analysis of this complicated fluid. We sometimes, however, find it deposited in the form of a yellow powder, in the gall-bladder, or concreted, with a little mucus, constituting a biliary calculus.

In this manner we have an opportunity of examining its chemical reactions. *Biliphæin* [italics added] is of a bright reddish-yellow colour, and is only slightly soluble in most fluids; it is devoid of taste and odour, and yields ammonia on dry distillation. Water takes up an extremely minute trace of biliphæin, just sufficient to communicate a faint yellow tinge. Alcohol dissolves more than water, but only a very inconsiderable quantity. Its best solvent is a solution of caustic potash or soda, both of which are more efficient than ammonia. On exposing this solution to the atmosphere, oxygen is absorbed, and the yellow colour becomes gradually green. On the addition of an acid to this yellow or green solution, there is a precipitation of green flocculi which possess all the properties of chlorophyll, or the green colouring matter of leaves. In this state it is termed *biliverdin* by Berzelius. It is no

[24] *Johann Franz Simon* (1807–1848) received the Dr. phil. in 1838 in Berlin, habilitated as *Privatdozent* at the Charité, Berlin in 1842.

longer *biliphæin* [italics added] (or *cholepyrrin* [sic] [italics added]), but a product of its metamorphosis.

The colouring matter of the bile may be separated from a composite animal fluid, by evaporation to dryness; by successive extractions with alcohol of ·845, ether, and water; by dissolving the colouring matter in a solution of potash, and then precipitating it, as biliverdin, by hydrochloric acid.

Diagnosis. The action of nitric acid affords a certain test of the presence of biliphæin.

b. After the separation of the *biliphæin* [italics added], by conversion into biliverdin, another colouring matter remains, to which Berzelius has given the name of bilifulvin. It is a double salt of lime and soda, combined with an organic nitrogenous acid, to which the term bilifulvic acid has been applied. When isolated, this acid is insoluble in water and in alcohol, and separates in pale yellow flocculi when it is precipitated from an aqueous solution of its salts by a stronger acid. Whether bilifulvin is an actual constituent of the bile, or whether it is a mere product of metamorphosis, is unknown.

Simon then went on to describe *Bilin*, which *Berzelius* considered to be "the principal and most important constituent of the bile," processing it, challenging it with chemicals and eventually digesting it with dilute hydrochloric acid to separate at least five components, including what turned out (later) to be such transformation products as taurine, and what *Berzelius* called fellinic and cholaric acids, both bile acids, as it turned out, *etc*. (*89*). And in his final summary on bile, *Simon* (*91*) described what to him was then the latest writings of *Berzelius* on the subject. In his own experience, though he was able to detect urea in blood, he was never able to detect the least trace of bile pigment (or *Bilin*) in the blood of a healthy calf. From which he concluded that *Bilin* was produced and secreted only by the liver. He restated *Berzelius'* findings on bile, as complicated and containing *Bilin*, *Cholepyrrhin* (or *Biliphäin*), biliverdin, cholesterol, sodium oleate, stearate and margarate, sodium chloride, sulfate, phosphate, sodium lactate, calcium phosphate, and, of course, mucus (*Schleim*). Other investigators (*48*) would add casein, ptyalin, carbonates, *etc*. to the list. *Simon* then went on to propose a separation scheme to permit quantitation of the components, especially the bile and salts, but *Berzelius* noted earlier that many of the isolated components may have arisen due to the methods and chemicals used in the separation.

Simon's own studies advanced in 1846 (*91*) included an analysis of morbid bile from the gallbladder of a man who died in a jaundiced condition. However, the analysis did not cite bile pigments, only red and black particles in suspension. The presence of bile pigment was indicated and summarized by *Simon*, however, due to the analyses of others: bile from a man with scirrhous pancreas (by *Chevallier*), bile from death due to cholera (by *Phoebus*), and bile from a man who died in a state of icterus (by *Scherer*). He then summarized the analyses of bile of animals, from *Berzelius*, *Gmelin*, *Thenard*, himself, and others.

Scherer[25] worked on the pigments of urine and separated a green pigment from the yellow-to-brown fresh urine of jaundiced patients and identified as the green pigment from bile on the basis of its color, solubility, properties, and reaction with HNO_3 (*92*):

[25] *Johann Joseph Scherer* was born on March 18, 1814 in Aschaffenburg and died on February 17, 1869 in Würzburg. He was a pioneer in clinical chemistry, graduated from the University of Würzburg, and after practicing medicine from 1831-1838, he studied chemistry in Munich, then in 1840 at Giessen with *Liebig* before returning to Würzburg in 1842 as professor.

> Der frische *Harn* ward zur Entfernung von Schleim und allenfalls schon ausgeschiedener Harnsäure filtrirt und hierauf mit Chlorbarium versetzt. Der erhaltene hellgrüne Niederschlag wurde sodann mit Wasser ausgewaschen, filtrirt und darauf aus demselben der Gallenfarbstoff nach zwei verschiedenen Methoden abgeschieden. 31

As further proof of the identity of his green pigment obtained from urine and the green pigment of bile, *Scherer* reported some of the early elemental combustion analyses of the two. After various manipulations of the green pigment from urine, he carried out elemental combustion analyses using lead chromate as the oxidant for producing CO_2 and H_2O, and the soda lime and hexachloroplatinate method for nitrogen analysis. He found no weighable ash, and reported the %C, H, N, O for two analyses (A and B). The data were compared with those (C) of a previously obtained sample of bile pigment (*Gallenfarbstoff*), but whether the difference in %C and %N was important or due to insufficient material could not be decided.

	A	B	C
%C	67.409	67.761	68.192
%H	7.692	7.598	7.473
%N	6.704	6.704	7.074
%O	18.195	17.937	17.261
	100.000	100.000	100.000

Scherer's early investigations of pigments from urine were later expanded, and a detailed separation method was outlined (*93*). *Scherer* assumed the right to call the pigment *Harnfarbstoff* (urinary pigment): ". . . so glaube ich mit Recht dieselben den namen *Harnfarbstoff* aufstellen zu dürfen, und werde sie dem nach der Kürze halber so benennen." He then presented the results (%C, H, N, O) of numerous elemental combustion analyses of the pigment isolated from the urine of individuals of varying degrees of health. While it is possible that *Scherer*'s *Harnfarbstoff* and biliverdin from *Gallenfarbstoff* were one and the same, the evidence at the time was only suggestive.

The French Revolution of 1789 subsequently inspired rebellion throughout the European continent, including the 39 cities of the German Confederation and led to the famous National Assembly of 1848 in Frankfurt. Though it passed a Basic Rights Law, the *Märzrevolution* failed in its purpose to meld German-speaking states, including those of the Austro-Hungarian Empire, into a German-speaking *Großdeutschland* confederation. It left behind *Kleindeutschland* under Prussian Hohenzollern leadership. The age coincided with the arrival of *Virchow*[26] who wrote a famous series of monographs (*Archiv für pathologische Anatomie und*

[26] *Rudolf Ludwig Karl Virchow* was born on October 13, 1821 in Schivelbein and died on September 5, 1902 in Berlin. He was known as the "Father of Pathology" and the founder of the social medicine field. He received the Dr. med. in 1843 in Berlin, was Professor at the University of Berlin until 1849, when he accepted the chair of pathological anatomy at Würzburg. In 1856 he returned to Berlin as Professor and established the *Virchow* Klinikum in eastern Berlin.

Physiologie) wherein, in 1847, he wrote an article on pathologic pigments (*94*). These he classified as three types: colored fats, altered or unaltered bile pigment (*Cholepyrrhin*), and altered or unaltered blood pigment *Hämatin* (hematin). Regarding *Cholepyrrhin*, *Virchow* wrote that it showed all the blendings from saffron yellow to dark brown to dark green, clearly not recognizing, as *Berzelius* did, that the colors represented distinct albeit related entities. From the physiological perspective, whether it was present in almost every tissue, it was found principally in the constituents of the biliary pathway. The pigment was associated with icterus and liver cells, during which condition the *Cholepyrrhin* collected in small, insoluble brownish or greenish grains that grouped into a nucleus (*94*):

> . . . Cholepyrrhin zeigt alle Uebergänge von Safrangelb durch das Dunkelbraun bis zum Schwarzgrünen, und obwohl es in fast allen Geweben vorkommen kann, so findet es sich doch am häufigsten in den die Gallenwege constituirenden Elementen. Jede Stauung der Galle in ihren Ausführungswegen bedingt zunächst eine Infiltration der um die Gallengänge gelegenen Leberzellen, einen partiellen Icterus (Hft. 1. pag. 159), so dass in allen Fällen, wo der allgemeine Icterus durch Gallenstauung bedingt ist, dem Icterus des Körpers ein Icterus der Leber voraufgeht. Die Infiltration der Leberzellen mit Cholepyrrhin ist zuerst eine gleichmässige, diffuse; sehr bald sammelt sich aber der Farbstoff in kleine, unlöslich, bräuniche oder grünliche Körper, die sehr häufig gruppenweise neben dem Kern liegen. 32

Virchow's article is written mainly from the perspective of liver pathologies and less from the chemical perspective, unlike the 1847 short paper (*95–97*) by *Heintz*.[27] *Heintz* addressed (*95–97*) what became known as the *Gmelin* reaction, in which bilirubin underwent a characteristic progression of color changes. He qualified it by indicating that HNO_3 did not produce the color change in every case in the presence of the components of bile. What had to be taken into account was that the reaction occurred not with the characteristic principal component of bile but with *Gallenbraun*, which *Simon* named *Biliphäin* in comparison to *Berzelius*' *Cholepyrrhin*. If HNO_3 brought about no color change, it was strongly assumed thereby that only the absence of *Gallenbraun* was proved, but not the absence of any other components of bile. However, the color change that HNO_3 caused in fluids that contained *Gallenbraun* was therefore at least a firm characteristic sign for the presence of this substance (*96*):

> Es ist bekannt, dass die Salpetersäure viel gebrauchtes Reagens ist, um die Gegenwart der Galle, in irgend einer Flüssigkeit nachzuweisen. Man giebt an, dass solche Flüssigkeiten dadurch zunächst grün, dann blau, violett, roth und endlich gelb gefärbt werden, und es ist diess in den meisten Fällen ganz richtig. Allein nicht in *allen* Fällen bewirkt die

[27] *Wilhelm Heinrich Heintz* was born on November 4, 1817 in Berlin and died on December 1, 1880 in Halle. He studied pharmacy in Berlin in 1840, was promoted to the doctorate in 1844 working under *Heinrich Röse*, habilitated in 1846 as *Privatdozent* at the Charité in Berlin and became a. o. Professor at the University of Halle-Wittenberg, then in 1856 *o. Professor für Chemie und Pharmazie* in 1855, and finally director of the pharmaceutical institute there. At Halle he supervised the doctoral work of *Johannes Wislicenus*, whose *pro-forma* advisor in Zurich was *Georg Karl Andreas Städeler* (Sections 2.8 and 2.9.1). He was the only chemist among the six founding members of the Deutsche Physkalische Gesellschaft.

> Salpetersäure bei Gegenwart von Gallenbestandtheilen jene Farbenveränderung. Zunächst muss berücksichtigt werden, dass, jene Reaction nicht durch die eigentlich wesentlichen Bestandtheile der Galle veranlasst wird, sondern durch das Gallenbraun, welches Simon Biliphäin, Berzelius dagegen Cholepyrrhin nennt. Wenn man also durch Salpetersäure keine Farbenveränderung hervorbringen kann, so ist, streng genommen, dadurch nur die Abwesenheit des Gallenbrauns, aber nicht die jener wesentlichen Gallenbestandtheile erwiesen.
>
> Allein jene Farbenveränderung welche Salpetersäure in Flüssigkeiten hervorbringt, die Gallenbraun enthalten, bliebe doch wenigstens ein sicheres Kennzeichen für die Gegenwart dieses Stoffs, wenn sie wirklich in jedem Fall einträte. 33

There is a certain logic to *Heintz*'s statements, if it actually occurred in every case. Yet it is unclear what problems this short paper clarified. In the various occasions when the *Gmelin* reaction had been employed from the time of its postulate by *Tiedemann* and *Gmelin* (*48*) in 1826 to 1847, it has been generally conceded that the color change reaction is diagnostic of bilirubin.

By the exact middle of the 19th century, the chemistry of bile pigments could be summarized rather simply. Bile of the mammals studied (man, ox, dog, *etc*.) was typically yellow, and the yellow pigment *Gallenfarbstoff* (a bile pigment) was difficult to separate by the available manipulations of the time, which were mainly extractions, precipitations as lead, barium, or tin salts, and washings. The liquids involved were typically water, ethanol, and ether. The pigment morphed easily into green (*Gallengrün*) during the separation, a color change that required (oxygen of) air. While this color change seemed to establish a 1:1 relationship between the yellow and green pigments, not all investigators agreed. A relationship between the pigments of bile and urine was investigated, with uncertain conclusions, while the relationship with the pigment of blood was an open conjecture. The *Gmelin* color reaction stood out as a sensitive analytical diagnostic for the yellow pigment. Combustion analyses were beginning to be employed, from which the %C, H, and N were determined for the bile pigment samples. However, the measurements were compromised by the formation of ash, which indicated impure samples. Even when the combustion left no ash, the samples were found to give different results after standing in air for a few days. Thus, *Lehmann* summarized the status of bile pigments in 1850 (*98*):

> Gallenfarbstoff.
> Chemisches Verhalten.
>
> Eigenschaften. Dieser Stoff gehört, wie so viele Farbstoffe, zu den chemisch noch sehr wenig erforschten Gegenständen; diess liegt theils daran, dass man sich denselben nur in sehr geringer Menge verschaffen kann, theils an seiner grossen Wandelbarkeit, indem er nicht nur im thierischen Organismus bereits unter verschiedenen Modificationen vorkommt, sondern auch bei der einfachsten chemischen Behandlung sich bereits umändert. Die gewöhnlichste Modification, welche auch die Ursubstanz der Gallenpigmente in den höhern Thieren zu sein scheint, ist das sog. *Gallenbraun*, *Cholepyrrhin* (*Berzelius*) *Biliphäin* (*Fz. Simon*). Dasselbe bildet ein rothbraunes, nicht krystallinisches Pulver, ohne Geschmack und Geruch, löst sich nicht in Wasser und sehr wenig in Aether, besser in Alkohol, der sich dadurch gelb färbt, in Aetzkali aber leichter noch als in Aetzammoniak; die hellgelben alkalischen Lösungen werden an der Luft allmälig grünlichbraun. Diese Modification des Gallenpigmentes ist es, von der die bekannten Farbenveränderung mancher gefärbter, thierischer Flüssigkeiten abhängen. Die gelbe Lösung dieses Pigments wird

bei allmäligen Zusatz von *Salpetersäure* (besonders wenn diese etwas salpetrige Säure enthält, *Heintz*) anfangs grün, dann blau (welches jedoch kaum bemerkbar ist, seines schnellen Uebergangs wegen in Violett) und roth; nach längerer Zeit geht die rothe Farbe wieder in eine gelbe über; dabei ist jedoch der Gallenfarbstoff völlig verändert. Durch *Salzsäure* wird derselbe aus der Kalilösung grün gefällt; dieser Niederschlag löst sich Salpetersäure mit rother, in Alkalien mit grüner Farbe auf, und scheint dadurch volkommen in die grüne Modification des Gallenpigments überzugehen. Der in frischer Galle enthaltene Farbstoff wird durch Säuren grün gefärbt; *Gmelin* fand, dass diese Färbung ohne Sauerstoffzutritt nicht statt finde; es ist daher höchst wahrscheinlich, dass die meisten jener Farbenveränderungen auf einer allmäligen Oxydation beruhen. Chlorgas wirkt auf dieses Pigment gleich der Salpetersäure, nur etwas schneller; grössre Mengen von Chlor bleichen den Farbstoff vollkommen und schlagen weisse Flocken nieder.

Dieses braune Pigment ist sehr geneigt, sich mit Basen zu verbinden, und zwar nicht blos mit Alkalien, sondern auch mit Metalloxyden und alkalischen Erden; auch mit letztern bildet es unlösliche Verbindungen, weshalb man den Stoff selbst oft für unlöslich gehalten hat.

Das *Gallengrün*, *Biliverdin* (*Berzelius*) is eine dunkelgrüne, amorphe Substanz, ohne Geruch und Geschmack, unlöslich in Wasser, in Alkohol wenig, in Aether mit rother Farbe löslich; Fette, Salzsäure und Schwefelsäure lösen es mit grüner, Essigsäure und Alkalien mit gelbrother Farbe auf. Beim Erhitzen wird dieser Körper ohne zu schmelzen und ohne merklich Ammoniak zu entwickeln unter Zurücklassung weniger Kohle zersetzt. *Berzelius* hält diesen Stoff für völlig identisch mit dem Chlorophyll der Blätter und glaubt auch alle 3 Modificationen des Chlorophylls in verschiedenen Gallen gefunden zu haben. Dieses grüne Pigment hat nicht mehr die Eigenschaft durch Salpetersäure Farbveränderungen zu erleiden; indessen findet man auch zuweilen grünliche Gallenpigmente, welche noch jene Eigenschaft besitzen. Meist schon nach der Behandlung mit Alkalien oder Säüren zeigt das Pigment der Galle andre Eigenschaften als der ursprüngliche Körper, theils wohl weil er mit diesen Stoffen selbst verschiedene Verbindungen eingeht, theils aber auch, weil er sich so leicht modificirt.

> Aus diesem Grunde sind die Angaben über die Eigenschaften dieser Stoffe so verschieden; man vergleiche *Berzelius* [1]), *Scherer* [2]), *Hein* [3]), *Platner* [4]) und Andre.
>
> *Berzelius* fand in der Galle auch einen Alkohol lösliche, in kleinen rothgelben Krystallen auschiessenden Stoff, den er *Bilifulvin* nennt. Ich habe denselben nur in Lösung, aber nicht in fester Gestalt erhalten können; auffallender Weise fand ich ihn oft in der mit neutralem und basisch essigsaurem Bleioxyd ausgefällten Galle, so dass er also durch diese Metallsalze nicht gefällt oder vielmehr im Ueberschuss des basischen Salzes wieder aufgelöst zu werden scheint.

Zusammensetzung. Bei unsrer Unbekanntschaft mit dem reinen unveränderten Gallenfarbstoffe ist es nicht zu verwunderm, dass seine elementare Zusammensetzung noch nicht bekannt ist. *Scherer* und *Hein* haben Gallenpigmente untersucht; allein es geht aus ihren Analysen hervor, dass sie sehr verschiedene Substanzen unter den Händen gehabt haben, und *Scherer* hat insbesondre gezeigt, dass das Gallenpigment durch Einwirkung von Luft, Alkalien und Säuren viel Kohlenstoff und Wasserstoff verliert. Man hat übrigens 7 bis 9% Stickstoff in dem Gallenpigmente gefunden.

Darstellung. Früher empfahl man gewöhnlich zur Darstellung des Gallenfarbstoffs, die aus solchem vorzugsweise bestehenden Gallenconcremente mit Wasser und Aether zu extrahiren; der Rückstand hat aber in der Regel nicht die oben angegebene Eigenschaft, sich in Alkohol zu lösen, da er mit Kalk in unlöslicher Verbindung ist (wie *Bramson* [1] [*Bramson*, Zeitschr. f. rat. Med. Bd. 4, S. 193–208] ganz richtig angegeben hat und jeder vorurtheilsfreie Beobachter sich leicht überzeugen kann), selbst in solchen Concrementen, die grösstentheils aus Cholesterin bestehen.

> Die *Bramson*'sche Untersuchungsweise, die ich oft widerholt habe, scheint mir gar keinen Zweifel an der Richtigkeit seiner Ausichten übrig zu lassen; übrigens stimmen damit auch die Gallensteinanalysen von *Schmid* [2] [*Schmid*, Arch. der Pharm. Bd. 42, S. 291–293]) und *Wackenroder* [3] [*Wackenroder*, ebendas. S. 294–296]) überein.

Berzelius stellt das Biliverdin aus der Rindsgalle dar, indem er den alkoholischen Auszug derselben mit Chlorbaryum fällt; der Niederschlag wird erst mit Alkohol, dann mit Wasser ausgewaschen und durch Salzsäure zerlegt, welche den Baryt auszieht; der Rückstand wird durch Aether von Fett befreit und dann in Alkohol gelöst.

Platner fällt den Gallenfarbstoff aus der Galle durch Digestion derselben mit Zinnoxydulhydrat; dieses bildet damit einen hellgrünen Niederschlag, der nach gehörigem Aussüssen mit Wasser mit schwefelsäurehaltigem Weingeist geschüttelt wird; aus der filtrirten grünen Lösung wird durch Wasser der Farbstoff in grünen Flocken gefällt.

Scherer schied aus gallenfarbstoffhaltigem Harn den Farbstoff durch Chlorbaryum aus, stellte ihn aber daraus auf 2 Wegen dar: entweder zerlegte er die Barytverbindung mit kohlensaurem Natron, und schlug aus der Natronlösung das Pigment durch Salzsäure nieder, wo es dann durch Auflösen mit ätherhaltigem Alkohol, Auswaschen mit Wasser u. s. w. gereinigt wurde, oder die Barytverbindung ward mit salzsäurehaltigem Alkohol extrahirt, die Lösung verdunstet, mit Wasser extrahirt und dann wie oben behandelt.

Prüfung. Ist die Gegenwart von Gallenfarbstoff in einer Flüssigkeit nicht zu gering, so giebt Salpetersäure, namentlich wenn sie etwas salpetrige Säure enthält, das oben erwähnte sehr characteristische Farbenspiel. Bei kleinen Mengen von Farbstoff giebt jedoch die Salpetersäure oft keine recht deutliche Reaction, so wie auch dann, wenn das Pigment schon zum Theil modificirt ist. *Schwertfeger* [1] [*Schwertfeger*, Jahrb. f. prakt. Pharm. Bd. 9, S. 375] empfiehlt in solchen Fällen die Flüssigkeit mit basisch essigsaurem Bleioxyd zu fällen, und den Niederschlag mit schwefelsäurehaltigem Alkohol zu extrahiren; dieser färbt sich bei Gegenwart des Pigments grün. *Heller* [2] [*Heller*, Arch. f. phys. u. pathol. Ch. Bd. 2, S. 95] räth der zu untersuchenden Flüssigkeit lösliches Eiweiss zuzusetzen, sobald sie nicht schon solches enthält, und dann durch überschüssige Salpetersäure zu präcipitiren; das coagulirte Eiweiss ist dann durch das Pigment bläulich oder grünlich blau gefärbt. Nach *Heller* bildet sich auf vorsichtigen Zusatz von Ammoniak zu Harn, der bereits umgewandeltes Gallenpigment enthält, wenn man nicht umschüttelt, auf der Oberfläche der Flüssigkeit eine rothe Scheibe.

Physiologisches Verhalten.

Vorkommen. Der Gallenfarbstoff findet sich in frischer Galle gewöhnlich aufgelöst vor, doch oft auch nur aufgeschlemmt; fast immer bildet er die Kerne zu Gallensteinen; zuweilen findet man auch ästige, knotige Concremente in der Gallenblase und den Gallengängen, die fast nur aus Gallenfarbstoff bestehen. Diesen Gallenfarbstoff hat man nicht blos in der Galle des Menschen und der Rinder gefunden, sondern auch in der andrer fleisch- und pflanzenfressender Thiere, jedoch in den verschiedensten Modificationen, wie schon die verschiedene Färbung der Galle nicht nur verschiedener Genera, sondern selbst verschiedener Individuen derselben Species lehrt; so ist die Hundergalle gelbbraun, die Rindsgalle bräunlich grün, die Galle der Vögel, Fische und Amphibien meist smaragdgrün. 34

In an age when no organic chemical structures could be proposed – or even imagined – there was a flowering of names for the pigments: *Gallenbraun*, *Cholepyrrhin* (by *Berzelius*) and *Biliphäin* (by *Simon*) for the yellow pigment; *Gallengrün* and *Biliverdin* (by *Berzelius*) for the green (*98*); and *Bilifulvin*, which *Berzelius* isolated from ox-bile as reddish-yellow (or orange) crystals and which were subsequently shown to give a positive *Gmelin* test. Soon to follow were two additional names for the yellow, and a scathing rebuke of the name *Biliphäin* by *Legg* (*99*):

The name cholepyrrhin is commonly said to have been given by Berzelius to the orange red pigment of the bile. F. Simon invented a barbarous word biliphæin, compounded of Greek and Latin, the use of which has been unfortunately endorsed by Heintz. Dr. Thudichum uses the word cholophæin, to avoid this bastard word. Städeler called this pigment bilirubin,

> forming the word with a cognomen like the other names which Berzelius used; biliverdin, bilifulvin, and the like. Maly has continued the use of the name cholepyrrhin.

Had *Legge* issued his rebuke some 20 years earlier, he might have persuaded investigators of the middle 1800s who followed *Simon* to drop the name. Yet *Biliphäin* persisted and, as will be noted in the following section, it was applied by *Heintz* to what was then the most highly purified bilirubin, apparently free from salts. Yet it eventually fell by the wayside, as did *Cholephäin*, in favor of the name *Bilirubin*. By the middle of the 19th century, coinciding nearly with the onset of the longest reigning (1848–1916) monarchy of Austria and its penultimate emperor *Franz Joseph I* (1830–1916) of the House of Habsburg-Lothringen, investigations of bile as a source of bile pigments began to wane, although not vanishing entirely (*100*). Rather, bile became the focus of studies directed toward its component bile acids and fatty acids (*85*, *86*, *101*). A more tractable source of the pigments, especially the yellow pigment, was proving to be gallstones, concretions found in the gallbladder or bile duct, as mentioned above in Section 2.1 and at the end of Section 2.4. For we now know that fresh bile contains little, if any, bilirubin, but numerous conjugates, such as bilirubin glucuronides, that are labile, sensitive to acid and base hydrolysis – and are poorly soluble in $CHCl_3$.

2.6 Bile Pigments from Gallstones and Urine, and Their Combustion Analyses during the late 18th to mid-19th Centuries

Gallstones, or concretions found in the gallbladder and in the bile duct, were recognized as such centuries ago (*102*, *103*). *Alexander Trallianus* (*Alexander of Tralles*, 525–605), the famous Greek physician mentioned concretions in the liver in his *Twelve Medical Books*, which were lost for a thousand years, then rediscovered and published in Paris in the year 1548 together with a Latin translation by *Stephanus*. Shortly thereafter, in 1549, *J. G. Andrenacus* dedicated a translation to *Thomas Cranmer* (1489–1556), Archbishop of Canterbury, 1532–1534, during the reigns of *Henry VIII* and *Edward VI*. In his translation of the second chapter of *Alexander*'s eighth book, which treats obstructions of the liver, it is found:

> Nam humores nimium exiccati assatique, lapidum instar concreverunt, adeo ut non amplius discuti potuerint. 35

referring to dried up humours, concreted like little stones and the cause of obstructions. However, even with gallstones having been found in humans and animals, and used as pharmaceuticals during the years following publication of the translation dedicated to *Cranmer,* little was known of their composition. They were described in detail regarding origin, size, macroscopic geometric structure, whether they floated on water, combustibility, texture, and color, which varied from white to livid yellow to green to reddish to blackish, in the case of humans, depending on the health of the individual. Gallstones were described as being friable and having

concentric (colored) layers. Yet from the middle of the 16th century and not until the 18th century does it appear that investigations of gallstones, though numerous, told much about the pigments contained therein. The investigations were largely of a medical and morphological nature for gallstones obtained from humans and wide variety of animals (*101–103*).

Coe (*29*), in 1757, was probably the first to publish a comprehensive monograph on the anatomical, clinical, and physical aspects of gallstones. He assumed that the concretions were formed in the gallbladder or bile ducts due to stagnation and inspissation from stopping or retarding the flow of bile. His comments on them did not go much beyond attempts to dissolve them in water and alcohol (see Section 2.1). Earlier in the 18th century, chemical investigations of gallstones were reported by *Vallisneri* (*34*) who described dissolving what must have been cholesterol gallstones in an alcohol-turpentine mixture, and other investigators [*Galletti* in 1748, *Haller* in 1764 (*35*)] reported on their dissolution, distillation and flammability. So that by the late 1700s, at the onset of the French Revolution (1789–1799), *Fourcroy* (*42*) was able to publish what may have been the first separation of pigments from colored gallstones, which had been pulverized and warmed in alcohol to dissolve steroids and other substances but which also leached out yellow-green pigments. And at the beginning of the 19th century *Thenard*, working with both bile and gallstones, found that yellow stones became green when exposed to air and that caustic alkali extracted a yellow pigment that gave a green precipitate upon acidification – thus achieving a partial separation of the bile pigment from gallstones (*53–56*) – and thereby providing an experimental link between the pigment of bile and that of gallstones.

Recall that one of *Thenard*'s most remarkable discoveries was nearly half a kilogram of a water-insoluble yellow powder in the bile duct of an elephant that died in the Paris zoo (*61*, *62*) in the 1820s. In the same decade when *Heinrich Heine* (1797–1856, one of Germany's most famous romantic poets) wrote the play *Almansor* in 1821 [with its famous line referring to the burning of the Qur'an during the Spanish Inquisition: "Dort, wo man Bücher verbrennt, verbrennt man am Ende auch Menschen" (portending the sequelae to the book burnings of 1933 Nazi Germany)], *Gmelin* in Heidelberg also achieved a separation of the yellow pigment of gallstones. This he accomplished by taking the pigment up into ammonium hydroxide, and found that the solution became green in air and precipitated in green flakes upon acidification (*48*). *Gmelin* not only demonstrated that the color change from yellow to green was due to oxidation, he also wrote of the series of color changes that accompanied the yellow pigment upon treatment with nitric acid – which became a standard analysis for the presence of the yellow pigment.

Fourcroy, *Thenard*, and *Gmelin* were not the only investigators of the pigment components of gallstones and bile during 1785–1826. In the mid 1830s, a *Monsieur Dr. Loir* in Paris also analyzed gallstones and classified them as: (A) those of pure *cholésterine* (cholesterol), (B) stones of cholésterine mixed with colored material, and (C) stones formed of colored material alone (nonflammable) (*64*, *65*). He indicated that *Thenard*'s picromel could be obtained from dried bile and describes brown-black, dark-green, sea-green, yellow and brick-red as colors of and within

concretions. And he concluded that the colored substance of bile appeared to be the only component, or the major principle of gallstones.

In the 1840s, *Berzelius* (*74*) described a method for isolating the pigments of gallstones, along with cholesterol and other substituents; however, the preparations were crude, hardly pure, and the isolation procedure tended to convert the yellow pigment at least partially to green. However, he was able to assert that the green pigment obtained from gallstones and bile was the same – and that both arose from oxidation of the yellow pigment (see Section 2.5).

In the same decade, *Scherer* (*104*), *Hein* (*105*), and *Bley* (*106*) published their findings on gallstones. *Scherer* reported finding a black-brown pigment in black gallstones that he purified by heating in ether, alcohol, and water (presumably separately). The final product gave ash upon incineration (41.79% C as $CaCO_3$ ash) and the following elemental analysis from combustion:

%	I	II
C	73.237	73.212
H	6.306	6.313
N	14.434	14.434
O	6.023	6.041

The %N was very high compared to later analyses of the pigments of gallstones.

In 1847 *Bley* (*74*) isolated what he believed to be *Biliphäin* from the gallstones of a deceased 60-year-old woman. (*Biliphäin*, the word coined by *Simon* (*89*) and rejected by *Legg* as bastardized (*99*) was rapidly picked up by *Heintz* (*95–97*) and used by him (*97*) for the purified *Gallenfarbstoff*/*Gallenbraun*.) Analysis showed the gallstone to be 96% cholesterol, with 4% a mixture of *Biliphäin, Gallenfarbstoff*, and gallbladder mucus. *Bley* noted that the stone contained both brown and reddish spots and a pale yellow core. After being pulverized it was yellowish in color and was extracted with 84% ethanol to leave a small brown residue. The last, upon treatment with aqueous KOH to effect dissolution, gave a positive *Gmelin* reaction. *Bley*'s was clearly a very qualitative experiment.

In contrast to *Bley*, *Hein* (*105*) gave a much more detailed analysis of gallstones, having at his disposal a large number of them, which he divided according to their color and (quantity) from ocher-brown (5), to black-brown (1), to brown (11), to yellow-gray (2), to yellow (15), to brown green (21). Samples were air-dried to remove water, burned to determine the amount of ash, and extracted with boiling ethanol to remove cholesterol and saponifiable fat. The last ranged from 8% to 85% by weight, with the black stone having the least. Following removal of the hot alcohol, a solid brown residue was obtained from every sample. It resembled the brown powder residue seen by *Berzelius* in a similar processing of gallstones following successive treatment with water, ethanol, and ether. *Hein* chose not to dissolve the brown residue in aqueous potash, as *Berzelius* reported (*72*), but to effect a partial dissolution using the weaker base ammonium hydroxide, to which he added hydrochloric acid to throw down a "sehr schön grünen Niederschlag" [very beautiful green precipitate], which fit the properties of biliverdin. The undissolved

brown residue (*Gallenbraun*) from ammonium hydroxide extraction corresponded to *Cholepyrrhin*. Thus *Hein* had apparently achieved at least a partial separation of biliverdin from bilirubin, but the samples were of questionable purity and the former may well have arisen from the latter during all of the extractions carried out in air. Indeed, the final *Gallenbraun* corresponding to *Cholepyrrhin*, when dissolved in aqueous potash, soon turned a lovely green color and, with added hydrochloric acid, yielded a dark green precipitate. The filtrate, still so deeply colored that light transmitted through it only at the edges, iridesced red-green. Redissolving the green precipitate in ammonia and reprecipitating with hydrochloric acid restored more brown than green. The brown material, heated in hydrochloric acid, dissolved as bright yellow, and the solution saturated with NH_3 was colored violet. Added hydrochloric acid threw down a very similar green precipitate. The brown material gave the characteristic *Gmelin* test and was submitted to combustion analysis, whose results are shown in Table 2.6.1. Thus a comparison of *Hein*'s first analysis (*Hein*-1) to *Scherer*'s brown pigment from gallstones (*Scherer*-1A, 1B) differ hugely in the %C, N, and O and especially in the amount of ash, with the latter being much richer. The percentages were determined after subtracting the ash content.

Table 2.6.1 Elemental combustion analysis data of *Hein*'s (*105*) *Cholepyrrhin* compared with *Scherer*'s (*92*, *93*) *Gallenfarbstoffe*

%	*Hein*-1	*Hein*-2A*	*Hein*-2B**	*Scherer*-1A	*Scherer*-1B	*Scherer*-2†	*Hein*-3A	*Hein*-3B
C	69.68	67.96	68.13	73.237	73.212	62.491	58.26	58.5
H	7.60	6.21	6.44	6.306	6.313	6.148	6.30	6.29
N	8.84	9.94	9.94	14.434	14.434	8.169		
O	13.88	15.89	15.49	6.023	6.041	23.122		
Ash	9.326	4.301	4.33	41.79	41.79	†	9.326	

* *bei der Verbrennung im Platinschiffchen* (from combustion in a Pt boat)

** *bei der Verbrennung zwischen Kupferoxyd* (from combustion mixed with CuO)

† *ohne Zahl für die Asche* (without accounting for ash)

In an attempt to reduce the amount of ash in the brown pigment, *Hein* processed his material by repetitive partial dissolution of *Gallenbraun* in ammonia or aqueous Na_2CO_3 followed by precipitation with HCl, with or without heating, treatment with alcohol, precipitation with basified acetic acid-lead oxide, then $BaCl_2$ induced precipitation followed by treatment with H_2SO_4, *etc*. – a longish, circuitous but historic processing of doubtful effect. Though the process reduced the amount of ash by roughly one-half and altered the %C, H, N, O somewhat (*Hein*-2A, 2B), the combustion analyses were still found lacking. Careful processing of yet another gallstone, after careful drying under vacuum with heating gave a much lower %C, H for ash-free material (*Hein*-3A, 3B). It thus seemed to *Hein* that the brown substance was not pure because it exhibited a variable composition depending on the method of isolation.

Hein also subjected the green material to combustion analysis (*Hein*-4, Table 2.6.2). This material, too, contained ash, but there was too little material for a

nitrogen analysis. These results may be compared with those from *Scherer*'s (*92*) green pigment that was isolated from fresh urine (*Scherer*-3A, 3B) and gave no weighable ash. Pigment from the same source, but which *Scherer* isolated using a different method gave slightly different results (*Scherer*-3C). As *Scherer* noted earlier, after the green pigment had undergone changes during long exposure to air, acid (*Scherer*-4A), and alkali (*Scherer*-4B), the %C and %H dropped considerably. The considerable variability in the analytical results is further illustrated in *Scherer*'s work (*92*) in which he stated in 1843 that the green pigment isolated from gallstones exhibited a high %C (*Scherer*-5) due apparently to a significant non-combustible component (as evidenced by the amount of ash). *Scherer* then reprocessed a small number of the same gallstones (apparently) to obtain an ash-free pigment, and he performed a new combustion analysis to give new results (*Scherer*-6) that were similar in %C and %H to those obtained from urine in *Scherer*-4A, 4B.

Table 2.6.2 Elemental combustion analysis data of *Hein*'s (*105*) biliverdin compared with *Scherer*'s (*92, 93*) *Gallengrün*

%	*Hein*-4	*Scherer*-3A	*Scherer*-3B	*Scherer*-3C	*Scherer*-4A	*Scherer*-4B	*Scherer*-5	*Scherer*-6
C	65.5	67.409	67.761	68.192	61.837	62.086	74	62.491
H	6.62	7.692	7.598	7.473	6.464	6.567	6.3	6.148
N		6.704	6.704	7.074	9.080	7.101	14.4	8.169
O		18.195	17.987	17.261	22.619	24.246		23.192
Ash	7.833							

It is clear from the studies of *Scherer* and *Hein* that just as elemental combustion analyses began to be performed somewhat routinely, so too were the pigments of gallstones, bile, and urine subjected to such analyses in the 1840s following isolation. Evidently, the isolation procedures did not always produce organic compounds entirely free of their salts or inorganic impurities, which led to the findings of ash left after incineration or combustion. Just how one factored out the ash may have led to the variable results seen in Tables 2.6.1 and 2.6.2. Such results did not serve to confirm that the green pigment isolated from gallstones, urine, or bile was in fact the same green pigment, or that the brown pigment isolated from the same sources was in fact the same compound. But clearly, by the middle of the 19th century combustion analysis had been introduced to bile pigments, and as such analyses continued during the following 50–75 years, the need for better methods of isolation and purification of the pigments became evident.

Shortly after the isolation and elemental combustion analyses of bile pigments from gallstones published by *Hein* (*105*) and *Scherer* (*104*), and from urine by *Scherer* (*92*), *Heintz* (*97*) studied the ash content of gallstones and bile pigments (*Gallenbraun*) isolated therefrom, noting the work of others (*Bramson*, *Schmid*, *Wachenroder*, and *Bley*, *Bolle*, *Scherer*, and *Hein*) and their analyses of the residue (ash) after combustion or burning. The ash was composed largely of calcium salts, especially CaO and $CaCO_3$, along with some $MgCO_3$. Isolating *Gallenbraun* from gallstones, *Heintz*, too, found considerable ash (9.41–9.91%) and considered that it

arose from impure pigment in which the calcium was bound not to carbonate but to the pigment itself. Repeating the experiment carefully with a different source of gallstones, *Heintz* concluded (*97*): ". . . dass in der That in dem rohen Gallenbraun wenigstens ein Theil des Farbstoffs mit Kalkerde verbunden ist" [that in crude *Gallenbraun* at least part of the pigment is bound to CaO].

He then sought to prepare the unbound pigment with an altered isolation procedure while taking special precautions to protect from air (oxidation) during each step of the manipulations (*97*):

> Nach den Versuchen von Gmelin geschieht diese Umänderung auf Kosten des Sauerstoffs der Luft. Um daher das Biliphäin, im reinen Zustande zu erhalten, muss jene Auflösung und Fällung in einem Raume geschehen, der keinen Sauerstoff enthält. 36

First, in an H_2-blanketed flask, crude *Gallenbraun*, previously washed (agitation) by hydrochloric acid and water, was dissolved in aqueous Na_2CO_3 as completely as possible by heating for a long time; then, it was rapidly filtered before dilute HCl was introduced into the dark brown-black filtrate, with CO_2 evolution, to separate dark brown flakes of *Gallenbraun* precipitate. The clear acidic supernatant was removed and allowed to stand, which precipitated pure *Gallenbraun* after washing with hot water and drying in air. The last, which *Heintz* called *Biliphäin*, possessed a dark brown color, tending toward olive green (*97*):

> Nachdem dieser Apparat zusammengestellt war, wurde mit der Wasserstoffgasentwickelung begonnen, und nachdem so viel dieses Gases entwickelt worden war, dass man annehmen konnte, auch in der im Kolben befindlichen kohlensauren Natronlösung befinde sich kein Sauerstoff mehr, wurde der Kolben geöffnet und schnell das mit Salzsäure und Wasser ausgewaschene rohe Gallenbraun hineingeschüttet. Nachdem sofort der Pfropfen wieder auf den Kolben gesetzt worden war, liess man mehrer Stunden Wasserstoffgas durch den Apparat strömen, bis auch in der Glocke sich kein Sauerstoff mehr befinden konnte. Darauf wurde die Natronlösung längere Zeit erhitzt, während der Gasstrom noch immer fortdauerte, und nachdem die Auflösung des Gallenbrauns möglichst vollkommen erreicht worden war, wurde das Rohr, welches die Gase aus dem zur Auflösung dienenden Kolben ableitete, so tief in diesen gesenkt, dass der Gasstrom die Gallenbraunlösung in die Glocke übertreiben musste. Hier wurde sie von dem darunter befindlichen Filtrum aufgenommen, und die klar davon abfliessende dunkelbraunschwarze Flüssigkeit filtrirte unmittelbar in die verdünnte Salzsäure hinein. Unter Kohlensäureentwicklung geschah die Zersetzung. Das Gallenbraun fiel in dunkelbraunen Flocken nieder. Nachdem die ganze Menge der Lösung auf diese Weise in die verdünnte Salzsäure klar abgeflossen war, wurde die Flasche herausgenommen, schnell umgeschüttelt, und mit einem Glaspfropf verstopft einige Zeit stehen gelassen, bis sie sich geklärt hatte. Der so erhaltene Niederschlag zieht nun nicht mehr so leicht Sauerstoff aus der Luft an, als seine Lösung. Er kann an der Luft mit heissem Wasser ausgesüsst werden.
>
> Im getrockneten Zustande bilden das reine Gallenbraun, welches ich Biliphäin nennen will, eine dunkelbraune, etwas ins Olivengrün ziehende Farbe. 37

Heintz described the solubility properties of his *Biliphäin* and indicated that in ammonia it forms brown precipitates, a calcium salt from $CaCl_2$ and a barium salt with $BaCl_2$. Dissolved in dilute alcoholic KOH, the solution rapidly turned green upon acidification by hydrochloric acid; then it changed to a beautiful blue color upon dropwise addition of HNO_3. Treatment of a dilute alkaline solution of *Biliphäin* with excess HNO_3 containing some aqueous HNO_2 gave the *Gmelin* color reaction characteristic of *Cholepyrrhin* (and bilirubin). Elemental combustion

analysis of *Biliphäin* revealed essentially no ash and the %C, H, N shown in Table 2.6.3. According to *Heintz*, the $C_{31}H_{18}N_2O_9$ (formula wt 562) molecular formula was a better fit than $C_{32}H_{18}N_2O_9$ (formula wt 574). However, the molecular weight of *Biliphäin* could not be determined experimentally at the time.

Table 2.6.3 Elemental combustion analyses of *Heintz*'s purified *Biliphäin* (I, II, III, IV) and the corresponding biliverdin (V) and calculated values for suggested molecular formulas (*97*)

%	I	II	III*	IV*	$C_{32}H_{18}N_2O_9$	$C_{31}H_{18}N_2O_9$	V	$C_{16}H_9NO_5$
C	60.70	60.71	61.06	61.03	61.94	61.18	60.04	60.38
H	6.05	6.02	6.09	6.06	5.80	5.92	5.84	5.66
N			9.12	9.12	9.03	9.21	8.53	8.80
O			23.73	23.79	23.23	23.69	25.59	25.16
Ash	0.29	0.37	0.045	0.079			0.11	

* *Biliphäin* from *Gallenbraun* separated from gallstones provided by Hrn. Dr. *R. Virchow*

The green product (*Gallengrün*/biliverdin) from air oxidation of *Biliphäin* was isolated and purified, and its elemental combustion analysis was obtained for the essentially ash-free pigment. *Heintz* compared the data (Table 2.6.3) to those corresponding to a molecular formula $C_{16}H_9NO_5$ (formula wt 295), although $C_{32}H_{18}N_2O_{10}$ (formula wt 590) would give the same %C, H, N, O. He wrote that his analyses for *Biliphäin* and biliverdin differed significantly from those obtained by *Scherer* (*92*) (Table 2.6.1) and *Hein* (*105*) (Table 2.6.1), noting that the first could not be correct due to the amount of ash and that anyway the method that *Scherer* used to isolate the pigment from icteric urine would not produce pure bile pigment. The pigment that *Hein* called *Gallenbraun* was separated from an insoluble residue from gallstones by heating crude *Gallenbraun* in ammonia. However, the differing results from *Hein*'s green material could also be attributed to an admixture with some fat or cholesterol, which explains both his 140–145°C melting point and his analysis. *Heintz* expressed hope that another chemist might repeat his experiments with suitable material to confirm or modify them. This wish was to be realized repeatedly during the following decades, but with bile pigments obtained from gallstones by somewhat different isolation methods (*97*):

> Die von mir für die Zusammensetzung des Biliphäins und Biliverdins gefundenen Zahlen weichen wesentlich von denen ab, welche früher von Scherer und Hein angegeben worden sind. Die des ersteren konnten aber kein richtiges Resultat geben, weil aus dem aus Gallensteinen dargestellten Farbstoff weder die Asche, die ja die kohlensaure oder kaustische Kalkerde enthalten konnte, noch das Epithelium entfernt worden war, und dass nach der Methode, welche er zu seiner Darstellung aus icterischem Harn anwendete, kein reiner Gallenfarbstoff erhalten werden könne, war *a priori* zu vermuthen. Die Versuche von Hein mit dem Körper, den er Gallenbraun nennt und den er durch Auskochen des rohen Gallenbrauns mit Ammoniak als unlöslichen Rückstand erhielt, trifft dasselbe, was gegen die zuerst erwähnten Versuche von Scherer gesagt worden ist. Der grüne Stoff aber, den Hein untersucht hat, muss eine zufällige Beimengung gehabt haben, da er bei 140°-145°C. schmolz. Wahrscheinlich enthielt er noch etwas Fett oder Cholesterin. Die Abweichung der Resultate seiner Analyse (er hat zudem nur eine Kohlenstoff- und Wasserstoffbestimmung ausgeführt) is demnach erklärlich. Es wäre zu wünschen, dass andere Chemiker, denen

> passendes Material, welches mir jetzt fehlt, zu Gebote steht, meine Versuche wiederholten um meine Schlüsse zu bestätigen oder zu modificiren. 38

It seems clear that despite the investigations of bile pigments up to 1850, there were very few advances toward what we understand as chemical structures. In 1850, chemical structure was still a remote or at least an evolving concept. The focus in the first half of the 19th century was on isolating the pigments from the natural sources, mainly from bile but also from gallstones, attempting to purify them (which was largely unsuccessful), making salts, and running combustion analyses. Perhaps all those efforts were a necessary precursor to the advances that were to come during the next 50 years. The latter was an era that also produced new and bizarre ideas (*e.g*. that bilirubin arose from the action of acids upon bile acids and amino acids), incredibly detailed isolation schemes, and a large number of pigment reactions, especially salt formation with ions such as Ag^{+}, Ba^{+2}, Ca^{+2}, *etc*. that revealed mainly that bilirubin is a diacid. The details that follow in Sections 2.8 and 2.9 reveal both a high level of experimental activity of a repetitious nature and a low level of actual breakthrough. Yet, possibly, it was a necessary step in the evolution of the structure of bilirubin. Perhaps the era represented "marking time" until organic chemistry had evolved to such a point where it might have a positive impact on "animal chemistry", bringing with it the concept of molecular structure and a level of synthesis "know how".

2.7 Bile Pigments from Gallstones in the Middle of the 19th Century

The late 1850s brought forth papers by *Charles Darwin* (1809–1892) and *Alfred Russell Wallace* (1823–1913) announcing a theory of evolution by natural selection in papers read at London's Linnean Society, and in 1859 *Darwin* published *On The Origin of Species*. Also brought forth was a breakthrough in bile pigment isolation and separation that was to have a lasting impact in the quest to determine the structure of bilirubin.

Soon after the work of *Heintz* (*95–97*), toward the end of the 6th decade of the 19th century, a new and improved method of pigment isolation from gallstones was introduced: extraction using chloroform. This rather remarkably simple alteration of the earlier established procedures (that involved washings with ethanol and ether, in which bile pigments are insoluble, and dissolving pigments in base followed by precipitation with acid or as barium or lead salt) also introduced a convenient way to separate the yellow pigment from the green, which was previously very difficult. In 1858, *Valentiner*[28] (*107*), while working in *von Frerichs*' laboratory in Breslau

[28] *Gabriel Gustav Valentin* (*Valentiner* = *Valentin* ?), 1810–1883, received the Dr. med. from the University of Breslau in 1832, studied with *Jan E. Puikyně* at the University from 1833, and from 1836 was Professor of Physiology at the University of Bern for 45 years.

and in Berlin, discovered that $CHCl_3$ digestion of pulverized gallstones, which had been exhaustively washed with ethanol and with ether to remove cholesterol, fats, and other solubles, produced a yellow $CHCl_3$ solution. Upon evaporation of the $CHCl_3$, while protecting the solution from air, red and red-brown crystals separated out, the majority (as he reported) with characteristics of *Hämatoidin*. As reported in *Virchow*'s *Archiv* (*108*):

> Herr Valentiner hat in dem Chloroform ein neues Lösungsmittel für thierische Farbstoffe gefunden. Zunächst gelang es ihm, aus gepulverten Gallensteinen nachdem er dieselben erschöpfend mit Alkohol und Aether ausgezogen hatte, durch Digestion mit Chloroform eine gelbe Lösung zu erhalten, aus der sich beim Verdampfen (unter Vermeidung zu starken Luftzutrittes) rothe und braunrothe Krystalle, der Mehrzahl nach mit den Eigenschaften des Hämatoidins, ausschieden. Es waren lancettförmige und rhomboidale Plättchen und prismatische Krystalle in drusiger Gruppirung. Um grössere Krystalle rein zu erhalten, war es vortheilhaft, der Chloroformlösung vor dem Verdunsten etwas thierisches Fett zuzusetzen und dies aus dem Rückstande rasch durch Aether auszuwaschen. Mehrmals wurde auch durch Aether-Auszug ein krystallinischer Farbstoff (Frerichs, Atlas zur Klinik der Leberkrankheiten Taf. I. Fig. 7) erhalten, sowie in vielen Fällen direct aus der Chloroform-Lösung Krystalle, die nach Farbe und Form von Hämatoidin verschieden zu sein schienen. 39

Although *Berzelius* had isolated red crystals (*Bilifulvin*) in 1840 from bile (*73–76*), apparently, red crystals from gallstones had not been isolated previously, and their color reminded one of *Hämatoidin* (hematoidin) (*Virchow*'s *Hämatoidin* (*94*), a crystalline or amorphous iron-free red pigment formed from *Hämatin* (hematin) in old hemorrhages).

Valentiner also described the extraction of hematoidin crystals from icteric liver or fatty liver and further described them in terms of shape and solubility. Prophetically, with respect to much later photochemical experiments, he reported that the crystals decompose over long exposure to diffuse daylight to give a porous, amorphous green powder. The hematoidin obtained by *Valentiner* was treated with conc. H_2SO_4 which decomposed it to green; concentrated aqueous KOH gave it a dirty red to green color; other reagents gave these and other colors. Significantly the hematoidin gave a positive *Gmelin* reaction. It would thus appear, on the basis of *Valentiner*'s extensive "tests" and manipulations that hematoidin and *Cholepyrrhin* or *Biliphäin* were the same substance (*108*):

> Ikterische, fettreiche Lebern, am besten die ikterische Fettleber höchsten Grades, bilden, bei Wasserbadhitze ausgeschmolzen, unter der sich abscheidenden Fettschicht und in den noch fettig durchtränkten Parenchymstückchen sehr zahlreiche Hämatoidin Krystalle. Es sind, auch wiederholter Reinigung und Umkrystallisrung gestreckte, fast rechtwinklige Täfelchen, denen bei beträchtlicher Dicke ganz flache Pyramiden, fast nur durch diagonal sich kreuzende Linien angedeutet, aufgesetzt sind. Bei Verunreinigung sind es gestreckte, rhomboidale Plättchen, zuweilen mit abgerundeten Winkeln, bisweilen dumbell-artig aneinander gesetzt, oder man sieht die bekannten schiefen Prismen mit rhombischen Endflächen, oder bei schneller Verdunstung feine rhombische Nadeln und kurze, fast rechtwinklige Tafeln. Die reine Substanz ist in Wasser, Alkohol und Aether unlöslich; in letzterem zerfallen die Krystalle, längere Zeit dem zerstreuten Tageslicht ausgesetzt, zu einen lockern, amorphen, grünen Pulver. Aetherische und fette Oele sind

> wirkungslos. Reine concentrirte Schwefelsäure löst unter raschem Farbenwechsel und Zersetzung zu körnigen flockigen Massen mit vorwiegend bräunlicher Färbung. Unterbricht man die Zersetzung durch Wasserzusatz während einer gleichmässig grünen Färbung, so erhalt man einen amorphen grünen Farbstoff, der durch Lösung in Ammoniak und widerverdunsten der Lösung in compacte grüne Körnchen und zarte formlose Häutchen geschieden kann. Salpetersäure (unreine) zersetzt rasch, unter anfänglich grüner, dann blaugrüner, blauer, endlich rothgelber und blassgelber Färbung bis zur Vernichtung jeder Farbe. Auch ein Gemisch von Salpeter- Schwefelsäure ruft den lebhaftesten Farbenwechsel der Gallenpigmentreaction hervor. Salzsäure giebt unter langsamer Zerstörung Dunkelgrün, schliesslich Blaugrün, jedoch lässt noch lange ein Zusatz von Salpetersäure die Masse chromatisiren. 40

In yet another experiment using $CHCl_3$ extraction, human and animal bile shaken with $CHCl_3$ always yielded hematoidin according to *Valentiner*. In addition to human bile, *Valentiner* investigated bile from the dog, cat, pig, cattle, sheep, chicken, goose, frog, and sturgeon and found that $CHCl_3$ extracted hematoidin and left behind dark green bile. In his experiments *Valentiner* thus discovered that the yellow-red pigment was soluble in $CHCl_3$ but the green was not, a distinction in relative solubility shared by bilirubin and biliverdin.

Very soon after *Valentiner*'s report, in 1859, *Brücke*[29] attempted to answer a question that he posed as to whether small amounts of bile pigment could still be detected after successful extraction using $CHCl_3$, *i.e.* how completely does $CHCl_3$ remove the pigment, with detection to be monitored using the very sensitive *Gmelin* color change reaction from HNO_3 (*109*):

> Im December vorigen Jahres machte Dr. Valentiner in Günzburg's Zeitschrift bekannt, dass sich aus Gallensteinen, aus der Galle, ferner aus den Lebern der Icterischen, oft auch aus anderen Geweben derselben mittelst Chloroform eine krystallinische Substanz erhalten lasse, welche verschieden von den bisher bekannten Gallenfarbstoffen sei und in allen ihren Eigenschaften mit dem Hämatoidin übereinstimme. Die chloroformige Lösung gab mit Salpetersäure in besonders schöner Weise die bekannte Farbenfolge der Gmelin'schen Gallenprobe; dagegen „enthielt nach Entfernung der in Chloroform löslichen Farbstoffe die immer noch stark dunkelgrün pigmentirte Galle kein Substrat der Gallenpigmentreaktion mehr". Dr. Valentiner schlägt desshalb vor, da, wo es sich darum handelt kleine Mengen von Gallenfarbstoff in einer Flüssigkeit nachzuweisen, diese mit Chloroform anhaltend zu schütteln und letzteres nach wieder erfolgter Trennung direct mit Salpetersäure zu prüfen. 41

In contrast to *Valentiner*, *Brücke* focused on extracting bile rather than gallstones. He found that $CHCl_3$ extracted a yellow pigment from human gallbladders. After separation by decantation, the $CHCl_3$ extract was placed in a retort and evaporated (without boiling) by heating on a water bath. The residue was then covered with spirits of wine containing 94% alcohol to produce crystals that partially adhered to the inside wall of the retort and partly sank as a brick red powder after shaking with the alcohol. After decanting the alcohol, the crystals were removed

[29] *Ernst Wilhelm Ritter von Brücke* (1819–1892) was a medical student in Berlin in 1838 and in Heidelberg in 1840. He was promoted to Dr. med. in Berlin and Professor in Vienna from 1848 to 1890.

from the retort by washing them out with alcohol and ether. *Brücke*'s examination (under a microscope) revealed that they were no longer co-mixed with extraneous material. He then proceeded to examine the residual, chloroform-extracted bile in order to determine whether the extraction had removed the bile pigment completely.

To settle this question, *Brücke* took a portion of the bile decanted after the initial $CHCl_3$ extraction and evaporated it to dryness. At this point one might wonder how complete the separation of bile from $CHCl_3$ was and whether $CHCl_3$ (with some solubilized bile pigment) was not dissolved in the bile. Of course that would perhaps have been difficult to determine in 1859. Though "separating" funnels had been known since the time of *Berzelius* (*110*), they would hardly be recognized as such today, and in the 1850s they were crude devices at best and not widely known. It was not until around 1854 that any device similar to a modern glass separatory funnel was employed. Irrespective, *Brücke* took the dried sample of $CHCl_3$-extracted bile, pulverized it, and digested the powder with $CHCl_3$, decanted the $CHCl_3$ and then added fresh $CHCl_3$ to the residue along with as much water as needed to dissolve the bile. The aqueous bile was then repeatedly extracted with fresh $CHCl_3$ and the color of the $CHCl_3$ extracts became weaker and weaker to imperceptible with each successive extraction. At this point it was presumed that the bile was completely depleted of the *Gmelin* test reactive bile pigment. However, the *Gmelin* reaction was still positive. Repeating the experiment, *Brücke* produced the same results (*109*):

> Abgesehen von einigen von Dr. Valentiner angegebenen Versuchen, welche ich mit den Krystallen anstellte, richtete ich meine Aufmerksamkeit zunächst darauf, ob in der That die durch Chloroform erschöpfte Galle die Farbenveränderungen mit Salpetersäure nicht mehr zeige. Ich dampfte einen Theil der von Chloroform abgegossenen Galle im Wasserbade zur Trockne ab, pulverte sie, extrahirte sie mit Chloroform, filtrirte dasselbe ab, leerte den Filterrückstand wieder in eine Flasche, übergoss ihn mit neuem Chloroform fügte dann wieder so viel Wasser hinzu, dass sich die trockene Galle darin löste. Nun extrahirte ich durch Schütteln weiter, indem ich das Chloroform von Zeit zu Zeit erneuerte; es nahm immer weniger Farbstoff auf, die Farbenveränderungen, welche es mit Salpetersäure zeigte, wurden immer schwächer und zuletzt unmerklich. Von der nun abgegossenen Galle wurde eine kleine Quantität mit vielem Wasser verdünnt, der Gmelin'schen Probe unterworfen und *zeigte den Farbenwechsel sehr schön*. Ich habe den Versuch mehrmals wiederholt und ihn theils in der ursprünglichen von Gmelin angegebenen Form angestellt, theils mit der Modification, welche ich vor zehn Jahren an dieser Probe angebracht habe und welche darin besteht, dass nur verdünnte Salpetersäure hinzugesetzt wird und dann concentrirte Schwefelsäure, welche sich zu Boden senkt und von unten her den Zersetzungsprocess einleitet, so dass man sämmtliche Farben gleichzeitig in über einander liegenden Schichten beobachten kann. Stets erhielt ich dasselbe positive Resultat. 42

Though *Brücke* found his results at odds with *Valentiner*'s description, a finding which begged explanation, he determined that the $CHCl_3$-soluble yellow pigment had all the characteristics of *Heintz*'s *Biliphäin* (or *Berzelius*' *Cholepyrrhin*) and was apparently analogous to *Virchow*'s hematoidin, as *Brücke* expressed (*109*):

> Diese Thatsache war in offenem Widerspruche mit Dr. Valentiner's Angabe, und es fragte sich, wie ich sie erklären sollte. Die durch Chloroform erschöpfte Galle bildete mit Wasser grüne Lösungen, dieselben wurden auch durch Zusatz von Kali nicht gelb, sondern

> nur ein wenig mehr gelbgrün, durch Salzsäure mehr blaugrün. Ich vermuthete desshalb, dass vielleicht von den beiden als Biliphäin und Biliverdin bekannten Farbstoffen, welche Object der Gmelin'schen Probe sind, der eine, das Biliphäin, in Chloroform löslich sei, der andere nicht, und es lag desshalb nahe, zu untersuchen, ob nicht die aus dem Chloroform erhaltenen Krystalle krystallisirtes Biliphäin oder doch eine krystallisirte Verbindung des Biliphains seien. Es würde diese ihre von Dr. Valentiner vertheidigte Identität mit dem Hämatoidin keineswegs ausschliessen. Virchow hat schon vor eilf Jahren auf die Analogien mit dem Biliphäin (Cholepyrrhin) aufmerksam gemacht, welche ihm sein Hämatoidin bei Einwirkung gewisser Reagentien darbot. 43

In order to resolve the apparent discrepancy with *Valentiner* regarding the persistent positive *Gmelin* reaction from *Biliphäin*-depleted bile, *Brücke* studied bile depleted of yellow pigment by $CHCl_3$ and found it to form a green solution with water that turned yellow-green upon addition of KOH, then blue-green with added hydrochloric acid. These colors were typical of biliverdin, which could be expected to remain in the bile after removal of *Biliphäin* by $CHCl_3$ extraction. And because biliverdin also exhibited a positive *Gmelin* reaction, the post-extraction bile would be expected to give a positive *Gmelin* reaction.

Probably even more important, *Brücke* found that while $CHCl_3$ extracted *Biliphäin* from bile, it did not extract biliverdin. That is, *Biliphäin* was soluble in $CHCl_3$ but biliverdin was not. *Brücke* also learned or knew that while *Biliphäin* was insoluble in alcohol, biliverdin was soluble. Thus, not only did the $CHCl_3$ extraction provide a route to pure *Biliphäin* from bile, or from a mixture of *Biliphäin* and biliverdin, but digestion of the latter with alcohol provided a route to remove biliverdin from *Biliphäin*. As a consequence pure biliverdin could be isolated following air oxidation of *Biliphäin*.

The salient points of *Brücke*'s investigations were succinctly summarized by *Virchow* in his *Archiv* in 1859 (*108*, *111*), thus bringing to a close the important discoveries from investigations of gallstones and bile at the end of the 6th decade of the 19th century (*111*):

> Herr Brücke wiederholte einen Theil der vorstehenden Versuche des Herrn Valentiner, zunächst um zu sehen, ob die durch Chloroform erschöpfte Galle keine Reaction mehr darbiete. Allein er fand, dass auch diese Galle bei der Gmelin'schen Probe den Farbewechsel schön zeigt, und es fragt sich nun, ob die erhaltenen Krystalle nicht Biliphäin oder eine Verbindung desselben seien. In der That erhielt er aus der ammoniakalischen Lösung der Krystalle durch Salzsäure gelbbräunliche Flocken, welche alle Eigenschaften des Biliphäins (Heintz) darboten, und aus denen sich durch Chloroform wieder eine gelbe Lösung und nach dem Abdestilliren des Chloroforms wieder Krystalle gewinnen liessen. Brücke schliesst daher, dass die neue Methode ein vortreffliches Mittel zur Scheidung von Biliphäin und Biliverdin sei. Letzteres lässt sich auch rein aus den rothen Krystallen gewinnen, indem man sie in wässerigem kohlensauren Natron löst und die Lösung an der Luft Sauerstoff absorbiren lässt, mit Salzsäure fällt, das Filtrat auswäscht und etwaige Reste von Biliphäin durch Chloroform auszieht. 44

Brücke repeated part of the preceding experiments of *Valentiner* [pp. 201–202 of the same *Virchow*'s *Archiv* (*111*)] to see initially whether bile that had been exhausted by $CHCl_3$ extraction can give any further reaction (specifically, the *Gmelin* test). He found that this bile, too, showed the beautiful change of colors of the *Gmelin* test. The question then arising was whether the (reddish) crystals obtained (following

evaporation of the $CHCl_3$ extracts) would not be *Biliphäin* or a compound of the same. In fact, on hydrochloric acid addition to an ammonia solution of the crystals, he obtained yellow-brownish flakes that presented the characteristics of *Biliphäin* (*Heintz*), and which dissolved in $CHCl_3$ to afford again a yellow solution from which crystals were able to be obtained upon distilling off the $CHCl_3$. *Brücke* concluded therefore that the new method was a superior means for separating *Biliphäin* and biliverdin. The latter could also be obtained pure from the red crystals by a process in which the crystals were dissolved in aqueous Na_2CO_3 and the solution allowed to absorb oxygen from air before HCl is added. The resulting (green biliverdin) precipitate was washed and any possible residue extracted with $CHCl_3$. Thus, the conclusions to be drawn from the work of *Valentiner* and *Brücke* were: (i) $CHCl_3$ is a superior solvent for removing *Biliphäin* or *Cholepyrrhin* from gallstones or bile following removal of cholesterol, fats, mucus, and other ethanol or ether-soluble components; (ii) $CHCl_3$ extraction leaves biliverdin behind; (iii) *Biliphäin* is soluble in $CHCl_3$; biliverdin is insoluble; (iv) biliverdin is soluble in ethanol; *Biliphäin* is insoluble; (v) pure *Biliphäin* can be separated from biliverdin by a $CHCl_3$ wash; and (vi) pure biliverdin can be isolated by extraction into ethanol following air oxidation of pure *Biliphäin*.

2.8 *Hämatoidin*, Bilifulvin, and the Origin of Bile Pigments

The biological origin of bile pigments remained a mystery for millenia until it began to unravel in the middle of the 19th century and in the absence of any knowledge of chemical structure. At that time it was known, of course, that yellow and green pigments could be isolated from bile and gallstones, whose colors ranged from light yellow to brown and blackish. *William Saunders* (1743–1817) speculated in 1809 that a relationship might exist between the pigments of bile and blood (*112*):

> Green and bitter bile being in common to all animals with red blood, and found only in such, makes it probable that there is some relative connexion between this third and the colouring matter of blood, by the red particles contributing especially to its formation.

Reddish crystals in old blood extravasations seem to have been noticed first by *Home* (*113*) in 1830 and subsequently in 1842 by *Rokitansky*, in 1843 by *Scherer*, in 1846 by *Zwicky* [as reported by *Wedl* (*114*) and *Robin* (*115*, *116*)], and in 1847 by *Virchow* (*94*). Yet despite the increasing investigations of bile pigments in the decades subsequent to *Saunders*, no further connection had been drawn between bile pigments and the red pigment of blood, which had been a separate focus of attention. That is until 1847, when *Virchow* reported extensively on the reddish crystals that he observed in extravasated or hemorrhaged (stagnant) blood from a very large number of diverse cases involving humans – and named *Hämatoidin* (hematoidin) (*94*):

> In Beziehung auf die Gefässe will ich die jenigen Fälle angeben, wo man am sichersten auf die Anwesenheit von Hämatoidin-Krystallen rechnen darf. 45

Hematoidin was described morphologically as to color (red), crystal shape, and dimensions. The red pigment of blood, named *Hämatin* (hematin), derived from the hemoglobin of red blood cells, is a protein-free reddish-brown crystalline solid obtained from dried blood. Of course, nothing was then known of its structure, but hematoidin was known to be different from hematin and suspected to be derived from it. *Virchow* credited an earlier investigator, Sir *Everard Home* (1756–1832) as having published (*113*) beautiful illustrations of clots from aneurysmatic sacs in 1830. Which left no doubt that he had seen genuine crystals of changed hematin (*94*):

> Die erste Beobachtung derselben finde ich bei Everard Home. In seinem letzten Werke (*A short tract on the formation of tumours. Lond.* 1830) sieht man auf der ersten Tafel 3 sehr schöne Abbildungen von Gerinnseln aus aneurysmatischen Säcken, welche keinen Zweifel übrig lassen, dass er wirklich Krystalle von verändertem Hämatin gesehen hat. Leider giebt er keine weitere Beschreibung davon, sondern bezieht sie nur auf Krystallisation von Blutsalzen (p. 22). In der Erklärung der Tafel heisst es: *The figure shows the different shades of colours of the layers, according to the length of time they had been deposited, and the crystallised salts as they appear in different parts of the coagulum.* 46

Virchow cited several other investigators, including *Scherer* (*104*), who had only a few years earlier reported finding red crystals in extravasated blood, and who linked that substance to his urinary pigment and the one from bile. A rather startling connection between blood, bile, and urine. *Virchow* described the red pigment as "Das pathol. Pigment, dass aus Hämatin stammt, kann also diffus, körnig und krystallinisch sein.… Es kann gelb, roth oder schwarz sein oder irgend eine Uebergangstufen. Zwischen diesen Farben ausdrücken." [The pathologic pigment that is derived from Hämatin can thus be diffuse, granular, and crystalline. . . . It can be yellow, red or black or possibly express a transitional state in between.] (*94*). But could a pigment found in blood (extravasations) be identical to a pigment found in bile? After many chemical probings of numerous and varied samples, including finding a positive *Gmelin* test from the action of hematoidin with H_2SO_4 + HNO_3, *Virchow* then concluded that "Eine Vergleichung unserer Pigmente mit den Gallenfarbstoff ist daher unabweisbar." [A difference between our pigments and the bile pigments is therefore irrefutable.] Yet, he repeatedly expressed an uncertainty as to whether his hematoidin was strictly identical to the brown bile pigment *Gallenbraun*, *Simon*'s *Biliphäin*, or *Berzelius*' *Cholepyrrhin*, both of which were separated from bile but had a rather different physical appearance and color and were of uncertain purity. Of major importance to physiology, might the origin of the bile pigments be interpreted as products of red cell consumption, as precipitated and altered hematin? If true, it would run counter to the conclusion of one of the most famous physician chemists of the times, for *Berzelius* had thought that green biliverdin (*Gallengrün*), which he knew was derived from yellow *Cholepyrrhin* by oxidation, was the same green pigment as that (chlorophyll) from green plants. And up to that time (and later), conversion of chlorophyll into the blood mass had never been confirmed. So *Virchow* rationalized the seeming contradiction by noting that, by *Berzelius*' accounts, biliverdin and not *Cholepyrrhin* was found only in the bile of herbivores (*94*):

> Kehren wir damit wiederum zu der Vergleichung unserer Pigmente mit dem Gallenfarbstoff zurück, so können wir die Bemerkung nicht unterdrücken, dass jeder Beobachter sich an

den einzelnen Fällen, wo ihm die Pigmente, namentlich das krystallinische, vorkommen, gewiss besser überzeugen wird, dass eine Ableitung derselben aus präformirtem Gallenfarbstoff nicht statuirt werden kann, als wir es hier durch lange Deductionen zu thun vermöchten. Die Unterschiede, welche wir zwischen beiden Arten von Farbstoffen aufgeführt haben, genügen nach dem jetzigen Stande der Chemie schon zu einer Unterscheidung, allein wenn man sie näher betrachtet, so wird man leicht einsehen, dass sie nicht bloss keine absoluten sind, sondern, genau genommen, mehr auf Verschiedenheiten der Cohäsion zurückführen, ja dass sogar eine ausserordentlich grosse Aehnlichkeit zwischen beiden Farbstoffen nicht weggeläugnet werden kann. Wir kommen damit auf eine andere Frage, die für die Physiologie des gesunden und kranken Körpers von der grössten Bedeutung ist, ob nämlich der Gallenfarbstoff als ein Produkt des Blutkörperchen-Verbrauchs, als ausgeschiedenes und verändertes Hämatin aufgefasst werden dürfe. Gegen diese Ansicht, welche von den verschiedensten Seiten seit langer Zeit, aber immer vollkommen hypothetisch, aufgestellt worden ist, schien namentlich die von Berzelius zu streiten, welcher die Aehnlichkeit desjenigen Gallenfarbstoffs, den er als Biliverdin bezeichnet, mit dem grünen Pflanzenpigmente, dem Chlorophyll hervorhob. . . .

. . . Die eigenthümlichen Farbenveränderungen, welche das bei Contusionen in die Hautgebilde extravasirte Blut eingeht, haben schon lange als Argument für die Umwandlung von Hämatin in eine dem Gallenfarbstoff ähnliche Substanz dienen müssen, allein man muss zugestehen, dass eine solche Art von Beweisen, wenn sie nicht einmal von einer wirklichen Untersuchung des Extravasates begleitet sind, gar nichts gilt. Die Frage wird aber von dem Augenblick an vollkommen erledigt sein, wo wir den Beweis exakt durch das chemische Experiment führen können, dass aus Hämatin nicht eine gelbliche oder grünliche Substanz, sondern eine dem Gallenfarbstoff identische entsteht. Ich schmeichele mir, dass die bisher mitgetheilten Untersuchungen den Weg zu einer endlichen Entscheidung der Frage angebahnt haben. Ich hätte gern diese Entscheidung selbst versucht, wenn meine zahlreichen Beschäftigungen mich nicht nöthigten, zu viel Gegenstände gleichzeitig zu verfolgen; mögen daher die vorstehenden Thatsachen anderen Beobachtern übergeben sein, um weiter verwerthet zu werden. Von einem besonderen Interesse erscheint mir dabei die Untersuchung der Bilifulvin-Krystalle. Könnte man aus der Galle Krystalle gewinnen, welche den im alten Blut entstehenden identisch sind, so bliebe nichts zu wünschen übrig. Die pathologische Anatomie scheint einen solchen Nachweis nicht möglich zu machen… 47

Virchow concluded, on the basis of a large number of observations that: ". . . ich die Wahrscheinlichkeit einer Umwandlung des Blutstoffes in Gallenfarbstoff bis zu einem möglichst hohen Grade gebracht habe." [. . . I have brought the likelihood of a conversion of the pigment of blood into the pigment of bile to the highest extent possible.] Also that the origin of jaundice (and its yellow color) is associated with changes in blood, especially the destruction of red blood cells: ". . . so scheint es vollkommen gerechfertigt, die Quelle der Gelbsucht in Veränderungen des Blutes und zwar speciell in einer ausgedehnten Zerstörung von Blutkörpchen zu suchen." [. . . thus it appears completely valid to seek the origin of jaundice from changes in blood, especially from the destruction of red cells].

In July 1853, the origin, status, and knowledge of hematoidin was summarized by the Austrian pathologist *Carl Wedl* (1815–1891) in Vienna (translated into English in 1855 by *George Busk* for the Sydenham Society) (*114*):

The *hematoiden* crystals of Virchow. Brilliant, transparent crystals, having the form of regular oblique rhombic prisms, and of a red colour, varying in tint and depth, according to the state of aggregation of the crystals. They are of a comparatively stable nature, and

> are insoluble in water, alcohol, ether and acetic acid. And they occur either free, or enclosed in flaky particles, or in cells, exclusively in extravasated blood, which has been retained for a longer or shorter time in the organism. . . . *Hematoidin* also occurs in the *amorphous* condition aggregated into reddish-brown granules or amorphous masses, mixed with crystals . . . Chemists have hitherto been unable to establish a theory of the formation of *hematoidin*, since the chemical composition of *hematin* itself is not as yet accurately determined, and that of *hematoidin* is still unknown.

In the mid-1850s, *Robin*,[30] while registering objection to the name *Hämatin* and preferring instead the name *Hämatosin* given first to it in 1827 by *Chevreul*, reviewed the history of hematoidin in 1856 and added his own extensive observations on its properties (*115*, *116*):

> Das in Prismen so wie das in Nadeln krystallisirte Hämatoïdin ist ziemlich hart, brüchig und bricht das Licht stark unter dem Mikroskop. Die Krystalle sind im Innern von lebhaft orangerother oder ponceaurother Farbe, an den Kanten und Ecken von dunkel carminrother Farbe. Im auffallenden Lichte haben die von allen Unreinigkeiten befreiten Krystalle eine dem Quecksilberjodid oder dem Alizarin ähnliche Farbe. Sie besitzen ein starkes Färbungsvermögen, sind etwas schwerer als Wasser und bilden voluminöse Massen. Die Winkel der Prismen sind 118° und 62°.
>
> An der Luft erhitzt entwickeln sie anfangs einen theerähnlichen Geruch, wie stickstoffhaltige Körper und brennendes Horn, entzünden sich alsdann und brennen mit leuchtender Flamme unter Zurücklassung einer aufgeblähten voluminösen Kohle, welche endlich vollkommen verschwindet. Es is deshalb die Verbindung schwierig im Verbrennungsapparat zu analysiren. Bei abgehaltener Luft entwickeln sich beim Erhitzen der Substanz übelriechende Gase, es destillirt eine theerartige Substanz und zurück bleibt ebenfalls eine voluminöse Kohle.
>
> Die Krystalle sind unlöslich in Wasser, Alkohol, Aether, Glycerin, ätherischen Oelen und Essigsäure, aber leicht löslich in Ammoniak. Die concentrirte ammoniakalische Lösung ist amaranthroth und nimmt bald einer safrangelbe und bräunliche Farbe an. In Berührung mit Kali und Natron zerfallen die Krystalle des Hämatoïdins und lösen sich allmählich auf, aber in geringerer Menge als in Ammoniak; die Lösung ist röthlich. Salpetersäure löst dieselben ziemlich schnell mit dunkelrother Farbe auf, unter Entwicklung von Gasblasen, wenn dieselbe concentrirt ist. Auch von Chlorwasserstoffsäure werden sie gelöst, aber in geringer Menge. Die Lösung ist goldgelb oder röthlichgelb; die ungelöst bleibenden Krystalle haben im auffallenden Lichte eine ockerbraune, unter dem Mikroskop eine röthlichgelbe Farbe. Von Schwefelsäure werden sie nicht gelöst; sie macht die Krystalle blos dunkler und nimmt eine grüne Farbe an, wenn die Krystalle noch Spuren von alkali- oder eisenhaltigen Verbindungen enthalten. 48

Though *Robin* looked at hematoidin from many angles, it is interesting to note that he reported no *Gmelin* bile pigment test on this pigment. He did, however, conduct an elemental combustion analysis on the crystals after attempting to remove all impurities by treating with water, alcohol, and ether, and (for comparison) he also analyzed *Hämatosin* (= *Hämatin*), for which he determined the formula $C_{44}H_{22}N_3O_6Fe$ on the basis of five analyses (*115*, *116*):

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[30] *Charles-Philippe Robin* was born on June 4, 1821 in Josseron and died on October 6, 1885 in Josseron. He was a biologist-physician and one of the founders of modern histology and member of l'Academie des Sciences de France.

Zur Analyse verwendete ich durch Wasser, Alkohol und Aether gereinigte Krystalle, nachdem ich mich zuvor unter dem Mikroskop überzeugt hatte, dass auf diese Weise alle Unreinigkeiten entfernt werden können und erhielt folgende Resultate:

	I	II	III
Kohlenstof	65,0460	65,8510	–
Wasserstoff	6,3700	6,4650	–
Stickstoff	–	–	10,5050
Sauerstoff	18,0888	17,1788	–
Asche	0,0002	0,00002	–

Hämatosin besteht im Mittel von 5 Analysen aus:

$C_{44}H_{22}N_3O_6Fe$

oder in 100 Theilen aus:

Kohlenstoff	65,84
Wasserstoff	5,37
Stickstoff	10,40
Sauerstoff	11,75
Eisen	6,64

49

The two hematoidin samples analyzed, with unusual accuracy, gave a bit of ash, determined to contain iron and traces of alkali salts, but no calcium, sulfur, or phosphorous. *Robin* compared the results of his analyses to those of *Mulder* (*117*) who reported removing iron from non-crystallizable hematin in 1839, and analyzing the product as 70.49% C, 5.76% H, 11.16% N, and 12.59% O, which gives the formula $C_{14}H_8NO_2$ – or as, *Mulder* wrote: $C_{44}H_{22}N_3O_6$. The first has the same composition as *Robin* found for hematoidin ($C_{14}H_9NO_3$, or $C_{14}H_8NO_2$+HO). *Robin* concluded that it was thus easy to recognize that hematoidin was nothing other than the pigment of blood, or a hematin, in which one equiv. of iron was replaced by one equiv. of H_2O (*115*, *116*):

Es ist deshalb leicht einzusehen, dass das Hämatoidin nichts anderes ist als der Farbstoff des Blutes, oder ein Hämatin, in welchem 1 Aeq. Eisen durch 1 Aeq. Wasser ersetzt ist. 50

By the late 1850s, *Valentiner* (*107*), the first to discover that $CHCl_3$ extracted nearly pure pigment from gallstones and bile, would find the brown-red crystalline residue from evaporation of the $CHCl_3$ extract to be suspiciously like hematoidin in its crystalline and chemical properties. At nearly the same time, in 1859, *Brücke* (*109*) came to a similar thought in connection with his extraction and purification of *Biliphäin* from bile. He noted, importantly, that the *Biliphäin* could be red and crystalline or amorphous and yellow depending on whether it had been obtained directly from $CHCl_3$ evaporation or whether it had been precipitated by acidification of an aqueous Na_2CO_3 solution using HCl. He also determined that the *Gmelin* test was positive for both *Biliphäin* (*Cholepyrrhin*) and the biliverdin derived from it by air oxidation. Such studies, even absent a knowledge of chemical structures, would suggest that *Biliphäin* and hematoidin were one and the same, or that

Hämatoidin contained *Biliphäin* (or *vice versa*) – and thus that bile pigments originated from the pigment of red cells, hematin.

Apparently, *Frerichs*[31] did not agree and was less certain – at least regarding the origin of the pigment of icteric urine. It was in Breslau that *Frerichs* published (in 1858) the first edition of his famous *Klinik der Leberkrankheiten*, volume I (*118*). In the preface (March 1858) he acknowledged the aid of two individuals, whose importance to bile pigments would become apparent subsequently: "Prof. G. Städeler of Zürich ('my friend Städeler'), who on many occasions aided Frerichs with chemical advice and carried out elemental combustion analyses of the abnormal transformation products found in the liver and urine, and Dr. Valentin(er) who performed a large part of the chemical work in Frerichs' lab." While in Berlin, *Frerichs* finished the revised second edition of 1861 (*119*) as well as volume II (*120*). In the former, he acknowledged the general acceptance that the hematin of blood was the origin of all pigments, and that it underwent metamorphosis into a yellow pigment (of jaundice) that was similar to or identical with a bile pigment (*119*):

> In neuerer Zeit, wo die Lehre von den Pigmenten eine sorgfältigere Bearbeitung fand und man sich mehr und mehr dahin einigte, dass das Hämatin des Blutes die Grundlage aller Pigmente ausmache, konnte es nicht an Beobachtern fehlen, welche nach der Idee von Senac icterische Färbungen der Haut, die, wie bei der Pyämie, bei putrider Infection und verwandten Processen, ohne Betheiligung der Leber sich enwickelten, auf eine directe Metamorphose des Hämatins zu einem gelben, dem Gallenpigmente ähnlichen oder mit demselben identischen Farbstoff zurückführten. 51

More controversial and seemingly contradictorily, *Frerichs* reported experiments from which he proposed that bile pigments originated from bile acids. This conclusion, especially when empirical formulas are taken into account, might seem far-fetched today. Nonetheless, given the state of chemical knowledge of the era, it was not irrational, though it was based almost entirely on the generation of colors and dubious results of the *Gmelin* reaction. His view in 1858 (*118*) and in 1861 thus rested on the work with *Städeler* and was expressed in the following (*119*):

> Diese Ansicht stützt sich auf folgende Thatsachen: Reine farblose Gallensäuren lassen sich in Gallenpigment umwandeln mit allen Eigenschaften, welche diesen Farbstoff auszeichnen. Eine solche Umwandlung erfolgt nicht bloss unter Einwirkung von Reagentien, sondern auch im Blute lebender Thiere, sie geschieht unter Aufnahme von Sauerstoff und

[31] *Friedrich Theodor von Frerichs* was born on March 24, 1819 in Aurich and died on March 14, 1885 in Berlin. He was professor of clinical medicine at the University of Berlin (Humboldt University of Berlin) and the founder of modern pathology. He studied medicine and science in Göttingen, learned chemistry from *Wöhler*, and departed in 1842 with a Dr. med. to establish himself as a surgeon of high repute and an ophthalmologist. In 1846, he returned to Göttingen, where he habilitated as *Privatdozent*, and in 1848, he was appointed a. o. Professor, working in association with *Wöhler* and *Rudolf Wagner*. He contributed to *Wagner*'s *Handwörterbuch der Physiologie*, and established himself as an excellent leader and researcher who expanded his expertise into clinical autopsies. He accepted a call as head of the academic medical institution in Kiel in 1850, then in Breslau (today's Wroclaw) as Ordinarius of Pathology and director of the medical clinic. In 1859, he succeeded *Schönlein* as director at the Medical Clinic at the Charité (Berlin).

ist zum Theil abhängig von dieser[1]). Durch Einwirkung von concentrirter Schwefelsäure bilden sich aus farbloser Galle Chromogene, welche an der Luft, und noch rascher unter Einwirkung von Salpetersäure, einen Farbenwechsel zeigen, volkommen übereinstimmend mit Gallenpigment. Dieselben Chromogene und Farbstoffe, welche sich ganz wie Cholepyrrhin verhalten, entstehen, wenn farblose Galle in reichlicher Menge ins Gefässsystem lebender Thiere injicirt. Die Gallensäuren werden in diesem Falle im Blute unter dem Einflusse der Respiration zu Gallenpigment umgewandelt. Dass solche Umwandlung auch die im Normalzustande aus dem Darm resorbirte und von der Leber direct ins Blut übertretende Galle erleidet, dafür scheint zunächst das reichliche Vorkommen von Taurin in der normalen Lunge, welches Staedeler und Cloëtta nachwiesen, zu sprechen. Die Pigmente, welche hierbei entstehen, treten indess erst dann mit dem Harn zu Tage, wenn der stetig weiterschreitende Umsetzungsprocess, welchem der Farbstoff unterworfen ist, schon eine Stufe erreicht hat, auf welcher er die Eigenschaften des Gallenpigments nich mehr besitzt.

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[1]) Wenn man vollständig entfärbtes reines glycocholsaures Natron mit concentrirter Schwefelsäure übergiesst, so bildet sich eine farblose harzähnliche Masse, welche in der Kälte mit safrangelber, beim Erwärmen mit rother Farbe sich auflöst. Aus dieser Lösung fällt Wasser farblose, grünliche oder bräunliche Flocken, je nach der Temperatur, bei welcher die Lösung erfolgte. Die durch Schwefelsäure veränderte Glycocholsäure hat die Eigenschaft, an der Luft rasch Sauerstoff aufzunehmen und damit in prachtvoll gefärbte Verbindungen überzugehen. Bringt man die durch Schwefelsäure entstandene amorphe, farblose Masse, nachdem sie möglichst von anhängender Säure befreit worden ist, auf ein Stück Filtrirpapier, so zerfliesst sie und es entsteht ein rubinrother Fleck, welcher bald blaue Ränder zeigt und nach kurzer Zeit indigblau wird. Nach einigen Tagen verschwindet auch diese Farbe und der Fleck wird braun.

Durch anhaltendere Einwirkung von Schwefelsäure auf Glycocholsäure wird eine Substanz gebildet, welche in Wasser mit tief grüner, in verdünnter Kalilösung mit brauner Farbe sich löst und auf Zusatz von Salpetersäure zuerst eine grüne, dann röthliche und zuletzt gelbe Färbung annimmt. Das Verhalten dieser Zersetzungsproducte gegen Salpetersäure erinnert an das der natürlichen Gallenpigmente, indess ist der Farbenwechsel weniger lebhaft. Ein mit dem Cholepyrrhin in jeder Beziehung sich gleich verhaltendes Product erhält man dagegen, wenn taurochalsaures Natron auf obige Weise behandelt wird. Mit wenig Wasser gelöst und mit concentrirter Schwefelsäure versetzt, färbt sich dasselbe prachtvoll roth und wird an der Luft allmälig blau. Vermischt man die roth gefärbte Lösung mit mehr Schwefelsäure, so geht die Farbe in braun über. Auf Zusatz von Wasser entsteht ein zarter, nach und nach blassgrün werdender Niederschlag; giesst man davon die säure Flüssigkeit ab und erwärmt den Rückstand, so treten intensiv grüne, blaue und violette Farben auf. Die gefärbten Producte lösen sich mit gallenbrauner Farbe in Kali, und die Lösung verhält sich gegen Salpetersäure vollkommen gleich einer alkalischen Cholepyrrhinlösung.

Dass dieselbe Metamorphose im Blute eines lebenden Individuums vor sich gehe, beweisen Injectionen von Auflösung entfärbter Galle in die Venen von Hunden. Der nach einem solchen Versuche gelassene Harn lässt beim Stehen gewöhnlich grüne Flocken fallen, welche auf Zusatz von Salpetersäure den für Gallenfarbstoff charakteristischen Farbenwechsel von Grün, Blau, Violett und Roth in schönster Form erkennen lassen. Unveränderte Gallensäure wird durch die Pettenkofer'sche Probe vergebens gesucht. Nur in einem Falle, wo eine ungewöhnlich grosse Menge, gegen zwei Drachmen, trockener Galle zur Injection verwandt wurde, liess sich eine Spur davon nachweisen. Bemerkenswerth ist, dass die Quantität des in den Harn übergehenden Farbstoffes am grössten erschien, wenn das betreffende Thier an Respirationsnoth litt, so namentlich bei einem Hunde, welcher in Folge des Versuches an Lungenödem zu Grunde ging. In einem Falle, wo eine geringe Quantität Galle injicirt war, das Thier auch frei von Athmungsbeschwerden blieb, wurde gar kein Pigment gefunden.

Or, as *Murchison* translated (*121*):

The bile-pigment is so intimately related on the one hand to the red matter of the blood, and on the other, to the colorless biliary acids, as to justify us in referring its origin to one or the other of these sources.

The intimate relation subsisting between the bile-pigment and the coloring-matter of the blood is indicated by facts which have been already mentioned, but more particularly by observations which have been recently made in my laboratory by Dr. Valentin (*Günsburg's Zeitschrift*, Dec., 1858), according to whom a portion of the coloring-matter of the bile dissolves in chloroform, and from this solution a crystalline substance may be obtained presenting all the characters of hæmatoidine. From this it appears possible, nay probable, that, as in extravasations, hæmatoidine may be developed from blood-pigment, so in like manner, in the vascular system and in the liver, the coloring-matter of bile may originate from the same source. Hitherto, however, no one has succeeded in obtaining bile-pigment directly from the red matter of the blood.

The second view rests upon the following facts:– The pure colorless acids of the bile may be transformed into bile-pigment with all the properties characterizing this substance. Such a transformation takes place not only under the influence of reagents, but it also follows the absorption of the acid substance (into the blood of living animals), and is in a measure dependent upon this.[1] By the action of concentrated sulphuric acid upon colorless

[1] If concentrated sulphuric acid is poured upon pure, perfectly colorless, glycocholate of soda, there is formed a resinous mass, devoid of color, which dissolves in the cold with a saffron yellow color, and with a reddish color upon the application of heat. This solution separates into a colorless water, and flakes of a greenish or brownish color, according to the temperature at which the solution has been made. Glycocholic acid, when changed by sulphuric acid, has the property, upon exposure to the atmosphere, of rapidly taking up acid substances, and of passing into gorgeously-colored combination. If the amorphous, colorless mass resulting from the action of sulphuric acid, after it has been deprived, as far as possible, of the adherent acid, is placed upon a piece of filtering paper, it dissolves, and there is produced a ruby-red spot, which soon presents a blue margine, and after a short time assumes an indigo-blue color. After some days, this color also disappears, and the spot becomes brown.

By the continued action of sulphuric acid upon glycocholic acid, a substance is produced, which dissolves in water with a deep green color, and in a weak solution of soda with a brown color, and which, upon the addition of nitric acid, assumes first a green, then a reddish, and lastly, a yellow tint. The behavior of this substance with nitric acid reminds us of that which characterizes the natural bile-pigment, although the change of color is less rapid. When taurocholate of soda is treated in the above manner, there is obtained in its place a product behaving in every respect the same as cholepyrrhin. When dissolved in a little water, and mixed with concentrated sulphuric acid, this assumes a brilliant red color, and gradually, upon exposure to the air, becomes blue. When the red solution is mixed with more sulphuric acid, the color passes into brown. Upon the addition of water, there is produced a delicate precipitate, gradually becoming pale green; if the acid fluid is pour off from this, and what remains is warmed, intense green, blue, and violet colors are produced. The colored products dissolve in potash, with a bilious brown color, and the solution behaves, with nitric acid, in precisely the same manner as a basic solution of cholepyrrhin.

That the same metamorphoses may take place in the blood of a living individual is proved by injections of colorless solutions of bile into the veins of dogs. The urine passed after such an experiment usually deposits, upon standing, green flakes, which, upon the addition of nitric acid, exhibit in a beautiful manner the alternation of green, blue, violet, and red colors, characteristic of bile-pigment. The unchanged acids of the bile may then be sought for in vain by means of Pettenkofer's test. In one case only, where an unusually large quantity (about two drachms of dry bile), was injected, a trace of it could be detected. It is worthy of notice, that the quantity of coloring-matter voided in the urine appears greatest, when the animal experimented upon has suffered from dyspnœa, as, for instance, in one dog, which died from œdema of the lung, consequent upon the experiment. In one case, where the quantity of bile injected was small, and the animal remained free from respiratory ailments, no pigment was found at all. The statements which have been made by Dr. Kühne (*Virchow's Archiv*, xiv., p. 810) in opposition to the correctness of this view, have been completely refuted by Dr. Neukomm (*Archiv für Anatomie und Physiologie*. Leipzig, 1820).

bile, there are formed color-producing substances (*Chromogene*),[2] which, upon exposure to the atmosphere, and still more rapidly on the addition of nitric acid, exhibit alternations of tints, corresponding in every respect with bile-pigment. The same pigments and color-producing substances (*Chromogene*), which in their properties precisely resemble cholepyrrhin, are produced by the injection of large quantities of colorless bile into the vascular system of living animals. In this case the acids of the bile are transformed in the blood into pigment under the influence of respiration. That the bile which has been re-absorbed from the intestine, or which has passed directly from the liver into the blood, may, under normal circumstances, experience a similar transformation, is an opinion which is favored in the first place by the presence of large quantities of taurine in the healthy lung, as shown by Staedeler and Cloëtta. The pigments, however, which are produced in this way, are not voided with the urine, until the constantly advancing process of transformation to which the coloring-matter is subjected, has gone so far, that the substance is no longer endowed with the properties of bile-pigment.

[2]Chromogen is a term applied by Frerichs to a colorless material which, when subjected to the action of certain agencies above mentioned, is transformed into the coloring-matter of bile. The relations of the two substances are somewhat analogous to those of colorless and blue indigo.—TRANSL.

Frerichs had thus conducted two experiments. One convinced him that colorless bile acids convert into bile pigments by submitting the former to cold conc. H_2SO_4 and observing a color change to saffron-yellow, and a reddish color upon heating. When diluted with water, greenish or brownish flakes appeared, depending upon whether the H_2SO_4 solution was kept cold or heated. When sodium glycocholate was treated with conc. H_2SO_4 variously colored combinations were observed. Prolonged treatment produced a substance that imparts a deep green color to water and a brown color in aqueous Na_2CO_3. Upon treatment with HNO_3, the color changes observed (green→reddish→yellow) were reminiscent of a slower reacting positive *Gmelin* test for bilirubin or biliverdin. From colorless sodium taurocholate treated with conc. H_2SO_4 the same result was observed, except that the product obtained behaved "in every respect like Cholepyrrhin".

Frerichs suggested that the same metamorphosis may take place in the blood of a living individual. The jump to this conclusion might seem far-fetched today. There was no evidence, other than color, that bile acids convert to bile pigments in conc. H_2SO_4, and there is no reason to believe that the pigmented material obtained from chemical transformation of a bile acid in H_2SO_4 might also be obtained when dissolved in blood. Nonetheless, the second experiment convinced *Frerichs*. He injected colorless bile acid salts into the veins of dogs and found that the voided urine usually deposited green flakes upon standing – and the green flakes gave a positive *Gmelin* reaction. However, he could find no unchanged bile acids in the

voided urine by examination using the *Pettenkofer*[32] test (*122*). Today, one might suspect that administration of bile salts intravenously would lead to lysis of red cells, leading to exposure of hemoglobin to be acted upon by heme oxygenase to yield biliverdin, and possibly biliverdin reductase to yield bilirubin – as happens in a hematoma.

Frerichs' two telling experiments received comments in the translator's preface in *Murchison*'s translation from German into English (*121*) of *Frerichs*' updated and revised volume I before publication of the latter (*119*). (In 1860 only the 1st edition of *Frerichs'* volume I had been published, but most of the additions and corrections for the 2nd edition were provided by *Frerichs* for the English translation.) *Frerichs*' conclusions regarding the origin of bile pigments from bile acids were both contested and supported, as in *Murchison*'s preface (*120*):

> This view as to the origin of Jaundice is supported by two experiments, tending to show that the colorless biliary acids may become converted into bile-pigment. 1. the coloring-matter of bile may be formed artificially out of compounds of the biliary acids with soda. If the glycocholate or tauro-cholate of soda be digested for a long time, at an ordinary temperature, with concentrated sulphuric acid, the solution gradually assumes several different colors, and after a certain time, on the addition of water, a flaky precipitate, resembling the coloring matter of bile, is produced. 2. Frerichs found that, on injecting ox-bile, entirely freed from its coloring-matter and mucus, into the veins of dogs, the urine afterwards secreted became deeply colored with a substance, which was ascertained on chemical analysis to be bile-pigment. None of the biliary acids injected were found in the urine, and, indeed, Frerichs denies that these acids are ever found in the urine along with bile-pigment, although they are sometimes present in urine having no jaundiced hue. From these experiments, which were repeatedly confirmed, it has been concluded, that there is an intimate relation between the biliary acids and the bile-pigments, and that in fact the former become converted into the latter when subjected to the influence of certain agencies; and it has been thought, that, under certain pathological conditions, the biliary acids normally present in the blood are transformed into bile-pigment.

In his preface written in 1860 (*121*), *Murchison* cited detractors to this theory, especially *Kühne*.[33] *Murchison* thus wrote, referring to *Kühne*'s work (*123*) that " … *Kühne* maintains that biliary acids do constitute an integral part of jaundiced urine, and he attributes the circumstance of their not having been hitherto demonstrated, to the insufficiency of the tests employed for the purpose." (*121*). More to the apparent controversy it generated (*121*):

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[32] *Max Joseph von Pettenkofer*, was born on December 31, 1818 in Lichtenstein and died (suicide) on February 10, 1901 in Munich. He was Professor of Medical Chemistry in Munich, who studied medical chemistry under *Liebig* in Giessen, and devised a test for bile acids involving heating in cane sugar and conc. H_2SO_4 to produce a purple coloration.

[33] *Wilhelm Friedrich Kühne* was born on March 28, 1837 in Hamburg and died on June 10, 1900 in Heidelberg. He was a respected German physiologist who studied under *Wöhler* and *Wagner* at the Universität Göttingen in the 1850s, following which he studied physiology in Berlin, Paris, and Vienna (with *K.F.W. Ludwig* and *E.W. von Brücke*) before taking charge of the chemical department of the pathological laboratory under *Virchow* in 1863. Some five years later, he was appointed Professor in Amsterdam, and in 1871 answered a call to succeed *H. von Helmholtz* at Heidelberg.

> Quite apart from the correctness of Frerichs' theory of icterus, which by the way, is only advanced as one that is highly probable, it is obvious that we have here to do with a question of facts, and the Kühne's facts are diametrically opposed to those brought forward by Frerichs. It is due, however, to Frerichs to state, that the results arrived at by him have been confirmed by several subsequent observers. Dr. Folwarczny, of Vienna (*Zeitschrift der kaiserl. u. königl. Gesellschaft der Aerzte zu Wien*. 1859. No. 15, p. 225), examined the urine in three cases of jaundice in Prof. Oppolzer's Clinique, but in all he failed to detect any trace of the biliary acids, although the examination was performed repeated, and Hoppe's process adopted in each case.
>
> Professor Staedeler of Zurich, and Dr. Neukomm, have likewise arrived at results similar to those of Frerichs, and have in Frerichs' opinion, completely refuted the statements made by Kühne. . . .
>
> . . . As to Kühne's opinion, that the coloring-matter which appears in the urine, after the injection into the veins of the colorless biliary acids, is derived from the hæmatine of the blood, it may be observed that, although it is possible that the coloring-matter of the blood may become transformed into bile-pigment, positive proofs are still wanting to show, that such a transformation really takes place. No one has yet succeeded in obtaining bile-pigment from the coloring matter of the blood. At all events, Kühne's experiments fail in proving that the coloring-matter in the urine originates from this source, and not from a transformation of the biliary acids; and they likewise fail in accounting for the disappearance of the biliary acids injected into the blood, in any other manner than that suggested by Frerichs.
>
> Further observations and experiments on the whole subject are still required; but in the meantime it should be understood, that the main facts adduced by Frerichs in support of his theory of Icterus have received confirmation at the hands of most subsequent observers.

Working in collaboration with, and thus supporting *Frerichs*, were *Städeler*[34] in Zürich, and *Valentiner* (in *Frerichs*' lab) and perhaps others. But what were *Kühne*'s facts that stood so clearly in opposition to *Frerichs*' theory? He reminded that bile acids hemolyzed red cells and proposed that the hemoglobin released is what leads to bilirubinuria, and that *Frerichs*' inability to detect intravenously administered bile pigments in urine was due to an insufficiency in the *Pettenkofer* test (*123–125*). In 1858, *Kühne* was, however, unable to show that intravenous injection of hemoglobin led to bilirubinuria.

Yet *Frerichs*' theory, his conclusions on the origin of urinary bile pigments, with its support from other scientists and relatively little dissent, would appear to have held sway in 1861. And these beliefs remained unaltered, even as studies by *Valentiner* in *Frerichs*' lab linked hematin to hematoidin, and hematoidin to bile pigments.

Despite his unaltered belief that bile pigments can arise from bile acids, *Frerichs* introduced new, potentially contradictory information in the updated and revised second edition of volume I (*119*). The information had emerged from studies in his own lab by *Valentiner*, who introduced $CHCl_3$ as an extraction solvent to remove

[34] *Georg Andreas Karl Städeler* was born on March 25, 1821 in Hannover and died on January 11, 1871 in Hannover. He received his doctoral degree in 1849 with *Wöhler* at Göttingen, was *Habilitant* and then a. o. Professor in 1851, before accepting a call as ordinarius Professor at the Polytechnic Institute in Zurich in 1853. (See Section 2.9.1).

Biliphäin (or *Cholepyrrhin*), which seemed from its color, crystal morphology, and *Gmelin* reaction to be the same as hematoidin or *Bilifulvin* (*71*, *109*). *Frerichs* considered it probable that the hematoidin found in blood extravasations was derived from the pigment of blood, and the pigment of bile might originate in the vasculature and liver from the same source (*119*):

> Der Gallenfarbstoff steht in so enger Beziehung auf der einen Seite zum Blutroth, auf der anderen zu den farblosen Gallensäuren, dass es gerechtfertigt erscheinen kann, seinen Ursprung von der einen wie von der anderen Quelle herzuleiten. Die nahe Verwandtschaft zwischen Gallenpigment und Blutroth ergiebt sich aus den bereits oben erwähnten Thatsachen, besonders aber aus den Beobachtungen, welche in neuerer Zeit von Dr. Valentiner in meinem Laboratorio gemacht wurden (Günsburg's Zeitschrift, Dec. 1858.), denen zufolge ein Theil des Gallenfarbstoffs sich in Chloroform löst und aus dieser Lösung beim Verdunsten in krystallinischer Form mit den Eigenschaften des Hämatoidins gewonnen werden kann. Hierdurch wird die Möglichkeit nahe gelegt, dass, wie in Extravasaten aus Blutroth Hämatoidin sich bildet, in ähnlicher Weise auch im Gefässsystem und in der Leber Gallenfarbstoff aus dieser Quelle hervorgehe. Bis jetzt ist es indess Niemandem gelungen, Gallenpigment direct aus Blutroth darzustellen.

Or as *Murchison* wrote in his preface to the English translation of *Frerichs*' second edition of volume I (*121*):

> Since the publication of the German edition of the first volume, certain experiments have been performed in Frerichs' laboratory by his assistant, Dr. Valentin [*sic*], which tend to show that one of the coloring matters of bile consists of hæmatine, the substance which is known to be derived from blood-pigment. Valentin has succeeded in detecting crystals of hæmatine in gall-stones, in the bile of men and animals, and in the tissues and secretions of jaundiced patients. The addition of chloroform is found to dissolve the hæmatine with a yellow color, and from this solution red and brownish-red, lancet-shaped, and rhomboidal prismatic crystals separate, which correspond in every respect with those of hæmatine (*Günsburg's Zeitschrift*, *Dec*., 1858). From these experiments, Frerichs admits there is an intimate relation between bile-pigment and the coloring matter of the blood, and even thinks it probable, that the former substance may be developed from the latter. Still he urges, that no one has succeeded in obtaining bile-pigment from the red matter of the blood, and that Valentin's results are not at all opposed to his theory of the convertibility of the colorless biliary acids into bile-pigment.

Shortly before the publication of *Frerichs*' 1861 updated 2nd edition of volume I of his *Klinik der Lebenkrankheiten* (*119*) and *Murchison*'s 1860 English translation of the greatly updated first edition (*119*), in 1860 (*126*), *Funke*[35] summarized work conducted with *Rudolf Zenker* on the origin of hematoidin from hematin derived from red cells. He indicated the identity of hematoidin with the *Biliphäin* obtained by *Valentiner* (*107*) and *Brücke* (*109*), and the *Bilifulvin* isolated first by *Berzelius* (*71*), then by *Virchow* (*94*). He concluded that *Bilifulvin* and *Biliphäin* were one and the same, and that the green pigment obtained by oxidation of *Biliphäin* is identical to the green pigment formed in stagnant blood. He cited *Kühne* as having carried out investigations showing that the bile pigment of icterus and icteric urine originated from the pigment of blood – contrary to *Frerichs*, who

[35] *Otto Funke*, born on October 27, 1828, died on August 17, 1879, was a German physiologist who was the first to crystallize hemoglobin.

believed the bile pigment of icteric urine came from metamorphosis of bile acids. *Funke* thought *Kühne*'s explanation superior: that bile acids injected into blood cause red cell lysis, and the red pigment so released is transformed into the bile pigment found in icteric urine (*126*):

> Der Ursprung des Gallenfarbstoffs lässt sich mit voller Bestimmtheit, wie der Ursprung aller im thierischen Organismus unter normalen oder pathologischen Verhältnissen sich bildenden Pigmente, auf den Inhalt der farbigen Blutzellen zurückführen, ohne dass wir nöthig haben, den vermeintlichen Farbstoff des Blutzelleninhaltes, das sogenannte Hämatin, als darin präformirt anzusehen. Wir haben bereits oben erwähnt, dass das krystallinisch aus der Galle dargestellte Biliphäin nach VALENTINER und BRUECKE sich mit dem Hämatoidin, jenem krystallinischen Umwandlungsproduct des Blutfarbstoffes, identisch verhält, während VIRCHOW früher schon beide Stoffe zwar noch als verschieden, aber doch als einander höchst ähnlich und in genetischer Beziehung zu einander stehend bezeichnet hatte. Ebenso evident geht diese Identität oder nächste Verwandtschaft aus einer früher gleichzeitig von mir und ZENKER gemachten Beobachtung hervor. VIRCHOW hatte unter pathologischen Verhältnissen in stagnirender Galle einen in geknickten Nadeln und Nadelgruppen krystallisirten rothgelben Farbstoff (FUNKE, *Atlas*, 2. Aufl. *Taf.* IX, *Fig.* 3) gefunden, welchen er „Bilifulvin" nannte, weil er ihn für identisch mit einem von BERZELIUS so benannten (dritten) Gallenfarbstoff hielt. Schon VIRCHOW machte auf die Aehnlichkeit dieses Bilifulvins mit Hämatoidin in seinem Verhalten gegen Reagentien aufmerksam; ZENKER und ich wiesen nach, dass Bilifulvin entweder von selbst oder durch Behandlung mit Aether sich in schöne grosse Krystalle verwandelt, welche alle Eigenschaften des Hämatoidins haben, also mit diesem identisch sind. Nehmen wir die oben genannten Beobachtungen von BRUECKE hinzu, so ist wohl nicht zu bezweifeln, dass dieses VIRCHOW'sche Bilifulvin nichts Anderes als krystallinisches Biliphäin ist. Kurz der normale braune Farbstoff der Galle, welcher durch Oxydation in den grünen übergeht, ist identisch mit einem in stagnirendem Blut sich bildenden Umwandlungsproduct des farbigen Blutzelleninhaltes. Einen weiteren trefflichen Beweis für den Ursprung des Gallenfarbstoffs aus Blutfarbstoff hat KUEHNE durch sein Untersuchungen über Icterus geliefert. FRERICHS hatte beobachtet, dass nach Injection von farbstofffreier Galle oder reinen gallensauren Salzen in's Blut im Harn Gallenfarbstoff erscheint, und hatte daraus geschlossen, das die Gallensäure sich im Blute in Gallenfarbstoff unwandelte, eine Metamorphose, welche er auch künstlich durch Digestion von gallensauren Salzen mit Schwefelsäure erzielt haben wollte. KUEHNE hat der genannten Thatsache eine andere besser gestützte Deutung gegeben. Die gallensauren Salz haben das schon oben erwähnte eigenthümliche Vermögen, die Blutkörperchen vollständig aufzulösen, daher auch nach Injection derselben in's Blut häufig zuerst blutig gefärbter Harn secernirt wird. Dieser aus den gelösten Blutkörperchen befreite Blutfarbstoff, nicht die Gallensäure selbst, welche neben dem Farbstoff im Harn erscheint, ist es, welcher sich in Gallenfarbstoff umwandelt, und so in den Harn übergeht. 52

Thus, in 1860–1861, a picture emerged that *Virchow*'s hematoidin was probably the same as *Berzelius*' and *Virchow*'s *Bilifulvin*, which appeared to be the same as the *Biliphäin* from *Valentiner* and *Brücke*. But the controversy over the origin of bile pigment in icteric urine was unresolved.

Why *Frerichs* conducted experiments to show that the origin of the bile pigment of icteric urine has its roots in bile acids is not entirely clear. Though it might seem an odd tangent, it should be recalled that *Frerichs*' earlier research on liver diseases and renal dysfunction involved the presence of leucine and tyrosine in urinary sediment in acute yellow atrophy of the liver – studies that may have had their origin in a youthful collaboration with *Städeler* involving studies of leucine and tyrosine

produced in humans and animals (*127*, *128*). The two probably met through *Wöhler* at Göttingen, where *Frerichs* was located from 1842 to 1850 and where *Städeler* received his doctoral degree in 1849. *Frerichs* and *Städeler* had clearly worked together in some fashion, starting in the early 1850s on leucine and tyrosine in biological tissues. By 1855, they had reported their findings on the presence of leucine and tyrosine in the human liver (*127*), a study possibly emanating from *Frerichs*' autopsies conducted in Kiel in 1851, wherein he found needle-like crystals in degraded liver cells from death due to liver atrophy and blood intoxication. Later, in 1853, while in Breslau, during an autopsy *Frerichs* found crystals in the hepatic vein of a liver with bile duct blockage, crystals that were separated and identified as leucine and tyrosine. (The bile was dark brown, with brown corn-like solids present). Treatment of tyrosine with conc. H_2SO_4 produced a red-colored solution from the dissolved solid. And it was stated (*127*) that a year earlier one of the authors (*Städeler*) had communicated to the *Köngl. Gesellsch. d. Wissensch. zu Göttingen* that tyrosine mixed with hydrochloric acid and sodium chlorate produced a red solution that turned yellow, with evolution of a gas. A connection between animal pigments and tyrosine should not be drawn from these experiments. But the authors noted that after injection of tyrosine into the blood system of an animal, it was not found in its urine, in contrast to leucine, thereby suggesting that perhaps the tyrosine was decomposed in the liver.

In 1856, *Frerichs* and *Städeler* published a follow-up paper (*128*) on the presence of leucine and tyrosine in animal organisms. Citing the earlier work (*127*) carried out prior to 1855, they indicated that proteins probably cleave in human organs as they do in the presence of acid or base to yield crystalline leucine and tyrosine that may accumulate in the liver in certain liver pathologies and which also (in the case of tyrosine) is used in the biosynthesis of bile acids. They found crystals of tyrosine in the urine of a woman with acute liver atrophy.

Given their interest in tyrosine, and the known fact that it could be produced from bile acids (taurocholic acid), it was not entirely illogical that *Frerichs* and *Städeler* might take an interest in bile acids and their metabolism. This led to their 1856 publication on the transformation of bile acids into pigments (*129*). It was known that while icteric urine is rich in pigment, it is devoid of bile acids, and these observations, reconfirmed by *Frerichs* and *Städeler*, led to the belief that there might be a close relationship between the bile acids and the bile pigments of urine. That is, with impeded bile flow the bile acids arrive either unaltered in urine or transformed into a bile pigment (*129*):

> Es kann als feststehend angenommen werden, dass in dem Harn Ikterischer, wenn derselbe reich an Pigment ist, keine Gallensäuren oder doch nur Spuren derselben vorkommen. Wir selbst konnten bei frühern wiederholten Versuchen keine Gallensäuren darin auffinden, gelangten also zu demselben Resultat wie Griffith, Pickford, Gorup-Resanez und Scherer. – Lehmann hat dagegen beobachtet, dass bei entschiedenem Ikterus in schwach pigmentirtem Harn die Gallensäuren oft in grosser Menge vorkommen.
>
> Diese Beobachtung, an deren Richtigkeit wohl nicht gezweifelt werden kann, schien uns entschieden darauf hinzudeuten, dass ein naher Zusammenhang zwischen den Säuren und den Farbstoffen der Galle vorhanden sei, und dass bei verhindertem Abfluss der Galle, die Säuren entweder unzersetzt in den Harn gelangen, oder zuvor im Blut oder irgend welchen Orangen eine Umwandlung in Farbstoff erleiden. 53

In order to examine this, it first had to be determined whether bile acids would convert outside the organism into pigments. And indeed, it was found that conc. H_2SO_4 dissolves sodium glycocholate (glycocholic acid is the amide of glycine with cholic acid) to afford a saffron yellow color that turns to a bright, fire-red-to-brown color upon warming. The glycocholic acid-turned-pigment took up O_2 from air to produce various colors. Precipitation by added H_2O produced flakes, which when gently heated turned violet, then blue after a few seconds. Similarly, filter paper coated with the aqueous H_2SO_4 solution and dried produced a green color (*129*):

> Wird reines glycocholsaures Natron mit concentrirter Schwefelsäure übergossen, so klebt es zu einer farblosen, harzähnlichen Masse zusammen, die sich in der Kälte mit safrangelber, beim Erwärmen lebhaft feuerrother bis bräunlichrother Farbe auflöst. Aus der Lösung fällt Wasser farblose, grünliche oder bräunliche Flocken, je nach der Temperatur bei welcher die Lösung erfolgte.
>
> Die durch concentrirte Schwefelsäure veränderte Glycocholsäure hat die Eigenschaft, an der Luft rasch Sauerstoff aufzunehmen, und damit in prachtvoll gefärbte Verbindungen überzugehen. Bringt man die durch Schwefelsäure entstandene farblose amorphe Masse, nachdem sie möglichst von anhängender Säure befreit worden ist, auf ein Stück Filtrirpapier, so zerfliesst sie, und es entsteht ein rubinrother Fleck, der bald blaue Ränder zeigt, und nach kurzer Zeit rein indigblau wird. Nach einigen Tagen verschwindet auch diese Farbe und der Fleck wird hellbraun. – Die Papiersubstanz scheint bei dieser Reaction ohne Einfluss zu sein, denn man beobachtet eine ganz ähnlichen Farbenwechsel beim Zerfliessen der amorphen Masse auf Glas oder Porzellan, nur tritt er in diesem Falle etwas weniger rasch ein.
>
> Die Lösung der Glycocholsäure in concentrirter Schwefelsäure enthält dasselbe Chromogen aufgelöst, die überschüssige Säure verzögert aber die Oxydation und die damit verbundene Färbung. Fällt man die Lösung mit Wasser, und erwärmt die von der sauren Flüssigkeit getrennten Flocken gelinde im Wasserbade, so färben sie sich nach wenigen Secunden violett und blau. Sehr schön beobachtet man auch den Farbenwechsel, wenn man ein Stück Filtrirpapier mit Wasser befeuchtet, dann mit der sauren Lösung bestreicht, und über der Lampe trocknet. Hat die Schwefelsäure, längere Zeit bei der Temperatur des Wasserbades auf Gallensäure eingewirkt, so wird der auf gleiche Weise auf Papier erzeugte Fleck grün. 54

To determine whether bile acids might become bile pigments, *Frerichs* and *Städeler* treated glycocholic acid with H_2SO_4 and were satisfied that colors were produced. The follow-up experiments were then directed to treating bile itself, decolorized and shown to precipitate substantial sodium taurocholate with added ethyl alcohol. This bile, which also contained other colorless components, when mixed with conc. H_2SO_4 turned red-brown with warming (likely due to heat of mixing) and reflected light with a vivid grass-green color. Exposure to oxygen of air turned the red-brown bile mixture to an indigo-blue color. The blue pigment separated as a solid mass upon addition of H_2O. The blue pigment partially dissolved to form a grass-green solution in alcohol and a green-blue residue, which turned greenish-brown upon dissolving in aq. potash. Treatment with acetic acid regenerated the original color.

Heating the original bile and H_2SO_4 solution for six hours produced substantially the same results, except the blue residue produced by addition of H_2O became yellow-green instead of green-brown upon partially dissolving in aq. potash. Addition of acetic acid gave the same green-brown color seen previously. In hot

acetic acid a bile-brown (*Gallenbraun*) color was seen, and this solution upon treatment with HNO_3, turned deep blue-green, then violet, then dirty yellow. This was vaguely reminiscent of the color display seen in the *Gmelin* reaction for bile pigments – a promising but misleading sign to the investigators. Treatment of the brown acetic acid solution (above) with $Pb(OAc)_2$ yielded a little colored precipitate that showed the (bile pigment-like?) display of colors upon treatment with HNO_3. At this point, these pigments (of uncertain purity and composition) and some of their solutions were beginning to behave like bile pigments toward the *Gmelin* reaction (*129*):

> Die syrupförmige Galle wurde mit dem 3-4 fachen Volumen concentrirter Schwefelsäure vermischt, wobei sie sich unter freiwilliger Erwärmung bräunlichroth färbte. Nach halbstündigem Erhitzen im Wasserbade war die Mass tiefer rothbraun und reflectirte das Licht mit lebhaft grasgrüner Farbe. Wasser fällte braune Flocken, die bei Luftzutritt erwärmt indigblau wurden. Die blaue Masse war in Wasser unlöslich, bei Siedhitze entstand eine braune Lösung, aus der sich beim Verdampfen ein Zersetzungsproduct als dunkelbraune Membran abschied. Die grasgrüne weingeistige Lösung des blauen Farbstoffs hinterliess beim Verdunsten einen grünlichblauen Rückstand, der beim Uebergiessen mit Kali gelbbraun wurde, ohne sich in wesentlicher Menge zu lösen. Säuren, selbst verdünnte Essigsäure, stellten die ursprüngliche Farbe wieder her.
>
> Nach sechsstündigem Erhitzen der Mischung von Galle und Schwefelsäure wurde im Wesentlichen dasselbe Resultat erhalten. Auch jetzt färbte sich die blaue Masse auf Zusatz von Kali gelbbraun, löste sich kaum im Ueberschuss, und ward auf Zusatz von Essigsäure wieder grünlichblau. Mit heisser Essigsäure entstand eine gallenbraune Lösung, die auf Zusatz von Salpetersäure sogleich tief blaugrün, dann violett und zuletzt schmutzig gelb wurde. – Essigsaures Bleioxyd erzeugt in der braunen essigsauren Lösung einen wenig gefärbten Niederschlag, der beim Uebergiessen mit Salpetersäure ebenfalls Farbenwechsel zeigte.
>
> Nachdem die Mischung von Galle und Schwefelsäure acht Tage lang auf einem mässig geheizten Wasserbade erhitzt worden war, hatte sich eine dunkelgrüne, aus kleinen mikroskopischen Kugeln bestehende Masse abgeschieden, die in saurem Wasser unlöslich, in reinem Wasser mit tief grüner Farbe löslich war. In verdünntem Kali löste sie sich vollständig mit rein gallenbrauner Farbe, und auf Zusatz von Salpetersäure trat zuerst grüne, dann röthliche und zuletzt gelbe Färbung ein.
>
> Das mitgetheilte Verhalten dieser Zersetzungsproducte gegen Salpetersäure erinnert an das der natürlichen Gallenpigmente, indess war der Farbenwechsel immer weniger lebhaft, wie man ihn beim Vermischen von stark pigmentirtem ikterischen Harn mit Salpetersäure beobachtet. Günstigere Resultate erhielten wir aber, als wir den amorphen, vorzugsweise aus taurocholsaurem Natron bestehenden Niederschlag, den wir mit Aether aus der weingeistigen Lösung der entfärbten Ochsengalle gefällt hatten, mit Schwefelsäure behandelten. 55

The authors were clearly impressed with the similarity in behavior (in colors, to some extent, and with apparently positive, or at least similar, *Gmelin* reactions) between natural bile pigments and the decomposition products which they obtained from bile acids. Though they were to be led astray by the correlation, they suspected that the pigments might arise as by-products of the biosynthesis of glycocholic acid – in which taurine ($^+H_3NCH_2CH_2SO_3^-$) is decomposed into glycine ($^+H_3NCH_2CO_2^-$) and *Saligenin* in the liver (*129*):

> Für jetzt beschränken wir uns darauf, auf die Aehnlichkeit der natürlichen Gallenpigmente mit den von uns erhaltenen Zersetzungsproducten der Gallensäuren aufmerksam zu

> machen; das aber glauben wir schon jetzt bestimmt aussprechen zu dürfen, dass das Chromogen, aus welchem durch Oxydation der blaue Farbstoff entsteht, mitunter in der Leber, und wie es scheint auch im Pancreas . . . vorkommt. Wir haben schon bei früherer Gelegenheit auf diesen Farbstoff aufmerksam gemacht, . . . damals war es uns aber noch unbekannt, dass derselbe in so einfacher Relation zu den Gallensäuren stehe. Auch der blaue Farbstoff, der sich mitunter aus Menschenharn auf Zusatz von Säuren abscheidet, und sich nach v. Sicherer's Versuchen in einen Körper umwandeln lässt, der dem Indigo vollkommen ähnlich ist, ist vielleicht ein Zersetzungsproduct der Gallensäuren. Wir sprachen schon früher. . . die Ansicht aus, dass dieser Farbstoff als Nebenproduct bei der Bildung der Glycocholsäure entstehen könne, indem sich das Tyrosin in der Leber in Glycin und Saligenen zerlege . . . 56

Which of course is a little far-fetched.

In a footnote to the 1856 paper (*129*) by *Frerichs* and *Städeler*, an experiment was cited in which a measure ("eine Drachme") of pure (?) colorless ox-bile dissolved in H_2O, was injected intravenously into a dog. Six hours later, 3 ounces of dark-brown urine were collected. It was strongly alkaline (the pH of urine is usually ~6.0). Upon standing, a thick sediment of green flakes precipitated which looked like brownish-green granules under a microscope. Addition of HNO_3 produced the most beautiful display of color changes characteristic of bile pigments. And the *Pettenkofer* reaction was negative (*129*):

> Neuere Erfahrungen haben aus diese allerdings bestätigt. Wir injicirten einem Hunde etwa eine Drachme reiner farbloser Ochesengalle, die in destillirten Wasser gelöst war. Sechs Stunden nachter liess das Thier gegen 3 Unzen dunkelbraunen Harns von 1,015 spec. Gew. und sehr schwach alkalischer Reaction. Beim Stehen liess derselbe eine ziemlich dicke schicht grüner Flocken fallen, welche unter dem Mikroskop als braungrüne Körnchen erschienen. Auf Zusatz von Salpetersäure zeigten sie auf das Schönste den für Gallenpigment characteristichen Farbenwechsel. Die Pettenkofer'sche Probe ergab ein negatives Resultat. 57

From the latter (hound) experiment, *Frerichs* and *Städeler* concluded that the origin of bile pigments (at least those excreted into urine) had their origin in bile acids. This apparently straightforward conclusion neglected the possibility that bile, or the bile acids therein, might have induced the release of a different pigment precursor from a component of blood, *e.g*. the "heme" of hemoglobin) from red cells (by cell lysis), which was converted to the bile pigment by way of hematin. The footnote supporting this contention is from work published in 1856 (*129*, pp. 105–106). *Frerichs* referred to the study in his 1861 second edition (*119*), and it was cited in *Murcheson*'s translation (*121*) published in 1860. In both publications, the work of *J. Neukomm* (thesis in Zürich, 1859) was indicated as support. *Neukomm* apparently worked in *Städeler*'s lab in Zurich and perhaps from this he also became associated with *Frerichs*. But what had *Neukomm* accomplished in Zürich that was so supportive of *Frerichs* and *Städeler*'s belief that bile acids were the source of urinary bile pigments in icterus? In March 1860, he published on the detection of bile acids in urine and their transformation in the blood stream (*130*). He took issue with *Kühne*'s experiments that showed bile acids injected into the bloodstream underwent no change and were expelled again in urine (*130*):

> W. Kühne hat gestuetzt auf eine Reihe von Versuchen, die Behauptung ausgesprochen, dass Gallensäuren, welche in die Blutbahn gelangen, keine Veränderung erleiden und durch den Urin wieder aus dem Körper entfernt werden. 58

It was the method of analysis and conflicting results associated therewith, *inter alia*, that was bothersome to *Neukomm*, and so he set about calibrating the *Pettenkofer* test with samples of ammonium cholate and sodium glycocholate, made up in urine, in order to compare the accuracy of the modification of *Hoppe* (*Hoppe-Seyler*, 1825–1895, see Section 2.10.2) used by *Kühne* to the usual method. In the usual method, according to *Pettenkofer*, a bile acid solution was mixed with 2/3 its volume of conc. H_2SO_4, after which a 10% solution of sugar was added with care and allowed to warm up to 70–75°C. Depending on the type and initial concentration of bile acid; for cholic acid at 0.4% a purple-violet coloration was observed, at 0.1% purple-red, at 0.04% weakly wine-red, at 0.01% weakly yellow. With glycocholic acid at the same concentration a noticeably weaker coloration was observed. A quantitative colorimetric experiment seemed to be required (*130*):

> Es sind hier indess nur die am besten gelungenen Färbungen angeführt, da auf dieselben raschere oder langsamere Mischung mit Schwefelsäure und die dabei unvermeidlichen Temperaturschwankungen von grossem Einfluss sind. Eine quantitative colorimetrische Bestimmung der Gallensäuren ist daher mit Hülfe der Pettenkofer'schen Reaction nicht zu erzielen. 59

From a series of careful quantitative measurements, *Neukomm* learned that the colors in the *Pettenkofer* reaction depended on the initial bile concentration and reaction temperature, irrespective of whether the H_2SO_4 was mixed rapidly or slowly. He concluded that quantitative bile pigment determination could not be attempted colorimetrically – a conclusion important to the level of bile acid detectability in urine (*130*):

> Die Grenzen der Reaction werden bedeutend erweitert, wenn man jenes Verfahren etwas abändert. Ich beobachtete, dass ein einziger Tropfen einer 1/20 procentigen Cholsäure oder Glycocholsäurelösung noch ein prachtvolles Purpurviolett liefert wenn man denselben in einer Porcellanschale mit einem Tropfen verdünnter Schwefelsäure (4 Theile HO + 1 Theil $HOSO_3$)[36] und einer *Spur* Zuckerlösung vermischt und unter Umschwenken über einer kleinen Spirituslampe vorsichtig und bei gelinder Wärme verdampft. Bei einigem Stehen der Probe nimmt die Farbe an Intensität ansehnlich zu. – Da 1 CC. nahezu acht Tropfen ausmacht, so gelingt es also auf diese Weise, noch 6/100 Milligrm. Gallensäure mit voller Schärfe nachzuweisen. Eine grössere Concentration der Lösung ist natürlich nicht störend; bei stärker Verdünnung hat man die zu prüfende Flüssigkeit zuvor auf einen oder zwei Tropfen zu verdampfen. – 1 CC. einer 1/100 procentigen Lösung beider Säuren gab auf die angegebene Weise noch die herrlichste purpurviolette Färbung, während bei gleicher Verdünnung und bei Anwendung von 3 CC. Lösung das Pettenkofer'sche Verfahren ohne Resultat blieb. 60

In the process of this experimentation, *Neukomm* devised a very sensitive modification to the original *Pettenkofer* test that effectively lowered the detection limit of bile acids in urine. Adding dilute H_2SO_4 to a small sample of urine, plus a trace of sugar, and warming to evaporation in a porcelain dish to display the colors (*130*):

> Gelang es nur auf die letzte Weise, das Vorhandensein von Gallensäuren zu constatiren, so wird diess in dem Folgenden der Kürze wegen durch „*Prüfung in der Porcellanschale*" angedeutet werden. 61

[36] The formulas, HO for H_2O and $HOSO_3$ for H_2SO_4, were based on *Gmelin's* atomic masses for H (1), O (8), and S (16).

To compare *Hoppe*'s variation of the *Pettenkofer* test, a clear solution of 0.1 g sodium glycocholate in 500 cc urine was mixed with milk of calcium, $Ca(OH)_2$, and heated to reduce the volume to ~2/3, then filtered, and the filtrate was reduced to a volume of ~50 cc. At which point excess HCl was added and the liquid was heated for ½ hour. It was strongly red-brown; to it was added 6–8 times its volume of H_2O to precipitate brown flakes. The precipitate was isolated and dissolved in alcohol; further processing afforded a yellow residue that was dissolved in a little aq. NaOH. This residue was submitted to the *Pettenkofer* reaction (adding H_2SO_4) to produce a reddish brown coloration that intensified upon addition of sugar – however without the characteristic color tone for bile acids. In contrast, when a portion of the solution was treated in *Neukomm*'s modified *Pettenkofer* test, a purple-violet color ensued. The same results were obtained from a 50% lower initial concentration of bile acid. On the basis of the unusual coloration in the *Hoppe* modification it was thought that that modification would lead to uncertainties in the case of ambiguous amounts of bile acids (*130*):

> Aus diesen Versuchen geht hervor, dass die Hoppe'sche Methode auch bei Anwendung nicht unbedeutender Mengen von Gallensäuren nur ein zweideutiges Resultat liefert und dass sie zur Nachweisung von kleinen Mengen ganz unbrauchbar ist. 62

In order to semi-quantitate his modified *Pettenkofer* test, *Neukomm* used lead (II) acetate to precipitate cholic acid and (separately) glycocholic acid from their aqueous solutions of ammonium cholate and sodium glycocholate. The precipitated lead salts were then converted back to small aq. volumes (3 cc) of sodium salts and treated with H_2SO_4 (2 cc) and some sugar to produce a purple-red color. The initial concentrations of bile salts ranged from 0.03 g/1,000 cc, to 0.005 g/1,000 cc, and in all cases the characteristic coloration was observed. Even at 100,000–200,000 times more dilute bile salt, the test was positive following isolation and processing of the lead salt precipitate.

These experiments established the great sensitivity of *Neukomm's* modification. In the same way he proceeded to analyze the same bile salts made up in urine to similar concentrations to yield the same colors and concluded that he could detect 0.001% glycocholic acid with his modification and only 0.02% using *Hoppe*'s – his being a factor of 20 more sensitive (*130*):

> Nach dieser Methode gelang es, 1/1000 pC. Glycocholsäure im Urin nachzuweisen, während dieses bei den nach Hoppe's Verfahren angestellten Versuchen bei 1/50 pC. kaum möglich war. Es ist daher jene Methode allein brauchbar, wenn es sich um die Nachweisung kleiner Gallensäuremengen handelt. Ja ich muss hinzufügen, dass die Hoppe'sche Methode in *allen* Fällen unsicher und daher untauglich zu sein scheint. 63

In order to sort out possible interference in the *Pettenkofer* test due to the presence of bile pigments, *Neukomm* investigated icteric urine. Here he showed the expected positive *Gmelin* reaction (for bile pigments), the display of colors from added HNO_3, and also a positive (modified) *Pettenkofer* test for strongly brown-colored icteric urine using the lead (II) acetate precipitation method, but a negative or highly uncertain test resulted from the standard *Pettenkofer* test (*130*):

> Da nun in der anfangs erwähnten Abhandlung W. Kühne eine Umwandlung der Gallensäuren im Blute ganz in Abrede stellt und behauptet, dass die demselben zugeführten

> Säuren durch den Harn wieder aus dem Körper entfernt werden, so schien es für die Physiologie sowohl wie für die Pathologie von Interesse zu sein, theils durch Untersuchung von icterischem Harn, theils durch Injectionsversuche an Thieren die Angaben Kühne's einer weiteren Prüfung zu unterwerfen.
>
> In dem Folgenden theile ich die Resultate der angestellten Untersuchungen mit.…
>
> Auch bei diesem Harn wurde also durch die gewöhnliche Pettenkofer'sche Probe ein negatives oder doch höchstens sehr zweifelhaftes Resultat erhalten, während nach unserem modificirtem Verfahren wenigstens Spuren von Gallensäuren unzweideutig nachweisbar waren. 64

With the preceding calibrations and controls accomplished, *Neukomm* advanced his studies to examining the urine of dogs injected intravenously with bile acids (aqueous sodium glycocholate). Following injection of the solution into a leg vein, urine was collected 12–15 hours (bright yellow) and again 36 hours (yellow) post injection. The first, weakly alkaline, became wine red upon the addition of H_2SO_4 and did not change upon addition of sugar solution. Crude concentrated HNO_3 showed at the contact point with urine a faint rose red ring without a tint of green. The second, acidic, behaved the same way with H_2SO_4 and HNO_3. Both urines were evaporated, taken up in alcohol and treated further as described earlier via precipitation by lead(II) acetate to produce the smallest hint of violet, which thus excluded the presence of bile acids in any considerable amount.

Four weeks later, the same dog was injected into the jugular vein with sodium glycocholate and urine was collected 15 hours, 26 hours, and 64 hours post injection. The first urine sample was dark brown and acidic (15 hours); the second was yellow and acidic with a tinge of dirty brownish green (26 hours); and the third (64 hours) was yellow and neutral. After standing several hours, the first urine yielded a greenish sediment with a positive *Gmelin* reaction. The greenish, yellow-brown filtrate, upon heating, gave red-brown flakes that did not dissolve upon addition of a little acetic acid. Filtration yielded a yellow filtrate and a greenish sediment. Added crude HNO_3 produced a barely perceptible *Gmelin* reaction. Addition of conc. H_2SO_4 showed a violet-red ring at the site of contact, and with complete mixing a wine-red color. Addition of sugar gave no further change. The second (26 hours) urine was yellow and acidic with a dirty brown-green sediment. Heating induced only slight turbidity that persisted upon increased acidification with acetic acid. Added HNO_3 produced a distinct *Gmelin* reaction; added conc. H_2SO_4 produced at the contact point a brown-red color, which in contrast to the urine lying on top, changed over to violet and blue.

The third urine (64 hours) was yellow and neutral, gave a barely detectable *Gmelin* reaction, and with conc. H_2SO_4 behaved as above.

The first and second urines were combined and divided into two equal parts. To one part an ethanol extract was prepared according to the earlier procedure. The other half was subjected to *Hoppe*'s method for detecting bile acids. From the ethanol extract, following processing, the lead salt was subjected to *Neukomm's* revised *Pettenkofer* test to give first a bluish color at the contact point with the lower H_2SO_4 layer, then violet and brownish. With complete mixing and addition of sugar it turned brown-yellow with a reddish tinge. In comparison, sodium glycocholate easily produced a purple-violet color. The *Hoppe* variation produced a

weak reddish-brown; added sugar turned it yellow-brown. Thus, no bile acid was detected in either version of the *Pettenkofer* test (*130*):

> In diesem Falle liessen sich also bei vorsichtiger Anwendung der üblichen Methoden keine Gallensäure im Harn nachweisen. 65

Fourteen days later the same, poor, overworked hound was again injected (jugular vein) with aq. sodium glycocholate, and yellow, weakly alkaline urine was collected 15 hours and 24 hours post injection. The first urine gave a negative *Gmelin* reaction with added HNO_3. Added conc. H_2SO_4 produced a weak violet-reddish to brownish color that did not change upon adding sugar. The second urine behaved in the same ways. The combined urines were split in half. One half was treated according to the modified *Pettenkofer* reaction, the other according to the *Hoppe* method, as above. Both methods gave the same results as above, a reddish-brown coloration with H_2SO_4 with no characteristic purple-violet color being produced even upon adding sugar.

The dog injection experiments were repeated with several different dogs, giving both similar and mixed results: sometimes an uncertain or negative test result for bile pigments; sometimes unequivocally positive. Usually, a negative result in the more sensitive modified *Pettenkofer* test seldom indicated the presence of bile acids.

Having gathered as much experimental evidence as he was able, *Neukomm* explained that: (1) while bile acids might have been found in icteric urine, the levels were too low to be detected in the usual *Pettenkofer* test; (2) in the animal experiments transfer of intravenously injected bile acids into urine was disproved by the usual *Pettenkofer* test and the absence of a bitter taste, as the facts proved that only traces of the injected bile acids passed into urine and that *Kühne's Pettenkofer* tests showing otherwise were deceptive (*130*):

> . . . In keinem Falle wurde aber ein bitterer Geschmack der schliesslich erhaltenen Natronverbindungen wahrgenommen; in keinem Falle liess sich darin mit Hülfe des gewöhnlichen Pettenkofer'schen Verfahrens Gallensäure mit einiger Sicherheit nachweisen, und nur in zwei Fällen wurde bei der Prüfung in der Porcellanschale eine charakteristische Färbung wahrgenommen.
>
> Diese Thatsachen beweisen, dass die ins Blut getretenen Gallensäure nur spurweise in den Harn übergehen können, und es wird damit der Ausspruch von Kühne: „*Die Natronverbindungen der Glycochol-, der Chol- und Choloïdinsäure verlassen in die Venen injicirt durch die Nieren den Körper des Thiers*" genügend widerlegt. Kühne hat sich mehrfach damit begnügt, direct mit den nöthigenfalls nur von Eiweiss befreiten Harn die Pettenkofer'scher Probe anzustellen; offenbar hat in solchen Fällen eine Täuschung durch die vorhandenen Farb- und Extractivstoffe stattgefunden, die, wie angeführt wurde, bei alleinigem Zusatz von Schwefelsäure zum Harn von Menschen und Hunden nicht selten zu rothen und selbst violetten Färbungen Veranlassung geben. 66

Neukomm noted the variability in the excretion of bile pigments or even traces of bile acids in the urine of dogs, post-injection, and that bile pigment was always seen by *Kühne*, along with "supposed" bile acid (*130*):

> Zuweilen enthält der Harn von Hunden, denen glycocholsaures Natron ins Blut injicirt worden ist, bald grössere, bald kleinere Mengen von Gallenfarbstoff. Frerichs . . . stellte 29 Versuche an, unter denen 19 ein positives Resultat gaben. Gewöhnlich enthielt dann der Harn gleichzeitig etwas Eiweiss und aufgelöstes Blutroth. Bei den von mir angestellten 7

> Injectionsversuch trat einmal der Farbstoff in solcher Menge auf, dass er sich zum Theil in Flocken ausschied, in zwei anderen Fällen war nur gelöstes Pigment vorhanden, die übrigen vier Versuche führten zu einem negativen Resultat. In den von Kühne mitgetheilten Versuchen war neben der vermeintlichen Gallensäure stets Gallenfarbstoff vorhanden. 67

The conclusion drawn was that injected bile acids probably led to the presence of bile pigment in urine, though injection might also have caused no production of bile pigment. Although *Kühne* completely denied such a transformation, he communicated that pigment regularly arose in post-injection urine, and he maintained that any bile pigment seen in such urine originated from the pigment of blood, the hematin released from red cells into blood. *Neukomm* did not agree with the last because when *Kühne* injected hematin no bile pigments appeared in urine, while when hematin and bile acids were injected together, bile pigment was detected in urine (*130*):

> Aus diesen von ganz verschiedenen Seiten gemachten Beobachtungen über Pigmentbildung bei Einführung von Gallensäuren ins Blut dürfte man schliessen, dass sich die Gallensäuren ebenso wie auf künstlichem Wege so auch in der Blutbahn in Chromogene und schliesslich in Farbstoffe verwandeln. Indess sind die beobachteten Ausnahmen nicht zu gering anzuschlagen; eine Umwandlung der Gallensäuren in Gallenpigment kann jedenfalls nur unter Zusammentreffen besonderer günstiger Umstände stattfinden. Mir wollte es scheinen, als ob dazu ein gewisser Grad von Irritation nothwendig sei, denn in drei von meinen Versuchen trat das erste Mal bei zufälliger, das andere Mal bei absichtlicher stossweiser Injection das Gallenpigment im Harn auf. Es fehlte an Hunden, um diese Versuche zu vervielfältigen.
>
> Kühne leugnet die Umwandlung der Gallensäure in Gallenfarbstoff gänzlich, obgleich er eine grosse Zahl von Versuchen mittheilt, bei denen regelmässig nach Galleninjection Pigment im Harn auftrat. Er vertheidigt die Ansicht, dass aller Gallenfarbstoff vom Blutfarbstoff abstamme, und zwar soll das beim Zerfallen der Blutkörperchen frei in Lösung gehende Hämatin ein Umwandlung in Gallenfarbstoff erleiden. Diese Ansicht erhielt aber durch das Experiment keine Stütze, denn als Kühne gelöstes Hämatin in die Venen injicirte, trat kein Gallenfarbstoff im Urin auf, während wenn er zur Injection gleichzeitig Hämatin und Gallensäure anwandte, die Bildung von Pigment beobachtet wurde. Kühne sieht sich daher auch gezwungen, der Gallensäure einen besonderen, noch räthselhaften Einfluss auf das gelöste Blutroth zuzuschreiben. 68

While *Neukomm* was forced to assume that blood was not the origin of the observed urinary bile pigment, he indicated that *Kühne*'s experiments did not disprove that bile acids injected into the bloodstream did not convert into bile pigments in the urine under certain circumstances (*130*):

> Ich bin weit davon entfernt anzunehmen, dass das im Körper zu Grunde gehende Blutroth *nicht* zur Bildung von Gallenfarbstoff Veranlassung geben könne, obwohl dieses durch das Experiment noch nicht nachgewiesen ist. Auf der andern Seite ist aber durch Kühne's Versuche nicht widerlegt worden, dass auch die in die Blutbahn gelangenden Gallensäure unter Umständen in Gallenpigment übergehen können. – Dass hier noch Lücken auszufüllen sind, ehe man diese Umwandlung als fest begründet betrachten darf, hat schon Frerichs ausgesprochen; häufigere Wiederholung der Versuche und vorurtheilsfreie Interpretation der erlangten Resultate wird uns allmälig zur Wahrheit führen. 69

One may summarize from the collection of experiments that bile pigments may sometimes be found in urine post-intravenous injection and that bile acids are usually not found. The simplistic conclusion would have been that injected bile acids

are converted into bile pigments. The less direct conclusion would be that injected bile acids induced the formation of bile pigments in urine. Had the chemical structures been known, one would have been forced to assume the latter.

Some ten years later, in 1871, *Edward R. Taylor* (*131*), a medical doctor, would write his prize essay, awarded by the American Medical Association, about the source of *Cholepyrrhin*: "Its *origin* has been pretty well made out to be from the hematin of the blood cell. … Virchow, in his Cellular Pathology remarks that hematoidine is the only substance in the body with which we are acquainted, that is allied to the bile pigment." Not a subject without lingering controversy, however, for *Taylor* noted that: "Frerichs contests the above views, and maintains that no one has succeeded in manufacturing bile pigment from the red coloring matter of blood. … On the contrary, he holds that biliary acids are the source of the bile pigments." By 1869–1871, however, experiments had been conducted which better explained *Frerichs*' experiments and reinforced *Kühne*'s thesis and thus laid to rest any doubt that *Cholepyrrhin* did not originate from hematin (*131*):

> . . . Niemeyer, however, holds the views of Kühne to be well established, for he says in the seventh edition (1869) of his practical medicine (I quote from Humphrey's and Hackley's translation), that the biliary acids "possess to a peculiar degree the property of dissolving the red blood-corpuscles. By injecting weak solutions of them into the blood of animals, we may artificially induce the so-called hæmatogenous icterus (jaundice without reabsorption), as the liberated coloring matter of the blood is transformed into biliary coloring mater. * * * * * The views regarding the occurrence of jaundice without retention and reabsorption of bile have totally changed since the observations of Virchow, Kühne, and Hoppe-Leyler [*sic*] have shown that bile coloring matter may be formed from the free coloring matter of the blood without the action of the liver; and we may induce artificial jaundice in animals by injecting substances that dissolve the blood-corpuscles. There is now no doubt that some of the formerly enigmatical forms of icterus are due to the disintegration of the freed coloring matter circulating in the blood, into bile coloring matter." Besides, the iron that both contain would point directly to a close kinship between them. It would seem, therefore, that we may finally rest upon the belief that the source of the cholepyrrhine [*sic*] of the bile is the hæmatin of the blood.

Though the relationship between bile acids and bile pigment seems now to be explained, the same could not be said with respect to hematoidin and its relationship to the bile pigments, for in the decade of the 1860s this issue was still one of considerable controversy. As noted earlier, *Zenker* and *Funke* reported (*126*) in 1860 that the red *Bilifulvin* pigment isolated from bile by *Berzelius* (*71*) changed into fine, large crystals, spontaneously or by treatment with ether, that were identical with hematoidin. As reported in 1862, *Jaffe*[37] (*132*, *133*), too, thought that hematoidin and *Bilifulvin* were identical, following his experiments on old brain hemorrhages, which he dried and extracted with $CHCl_3$ and evaporated to yield golden yellow crystals that showed a positive *Gmelin* test. As with *Jaffe*'s work,

[37] *Max Jaffe* was born on July 25, 1841 in Grünberg and died on October 26, 1911 in Berlin. After his early education in Grünberg and Breslau, he received the Dr. med. at the University of Berlin in 1865 and became *Assistent* to *Leyden* at Königsberg, where he later became Professor of Pharmacology.

which to him proved the existence of a bile pigment not originating from bile, *Hoppe-Seyler* (*134*) isolated the same pigment, with the same properties from a cyst in the breast. *Städeler* (*135*) criticized the crystal morphology cited by *Valentiner* (*107*, *108*), finding the crystal angles of the orange-colored elliptic, *i.e.* crystals (of bilirubin) isolated following evaporation of $CHCl_3$, as being very different from those of hematoidin, which never had convex surfaces. *Holm*, working in Zürich with *Städeler* (*136*) noted that the yellow $CHCl_3$ extract of hematoidin from old hemorrhages of the brain turned green upon exposure to light (a characteristic of bilirubin). His hematoidin from corpora lutea of cows as well as brain hemorrhages differed from bilirubin in crystal form, color, and solubility properties in ether, CS_2, and alkali. In comparing hematoidin from a cyst to bilirubin, *Salkowski* (*137*) found somewhat different results from *Holm*: similar crystal forms, solubility, and positive *Gmelin* test. In contrast, *Preyer* (*138*), in 1871, strongly expressed his opinion (on the basis of his spectral measurements) that hematoidin and bilirubin are not identical. (Spectra at the time were measured by the absence or diminution of certain regions of the visible spectrum when visible light was passed through a dissolved sample of pigment.) To complicate matters further, *Thudichum* (1829–1901, see below, Section 2.9.2) claimed that the others had examined not hematoidin but *Lutein* (lutein, also xanthophyll) – and that lutein differed altogether from bilirubin. At essentially the same time, *Kühne* (*123–125*) and *Hoppe-Seyler* (*134*) began to use hematoidin as a synonym for bilirubin.

The dispute over the origin of bile pigments, whether from bile acids or otherwise, was nowhere as long lasting as the dispute about the identity of hematoidin with bilirubin. The controversy over the identity of hematoidin did not slip easily away. In the sixth edition of his *Manual of General Pathology* (English translation published in 1876) *Ernst Wagner* wrote that blood extravasations consisted of not just hematoidin but also contain bilirubin (and probably other compounds). Following *Thudichum*, *Wagner* preferred to call the pigment lutein rather than hematoidin. He cited the studies of *Holm* and *Städeler* (*136*), who believed to have shown that hematoidin was not identical to bilirubin (*139*):

> Hæmatoidin was for a long time considered identical with bilirubin, and because bilirubin (from bile) like hæmatoidin (from extravasations of blood) separated from its solutions in chloroform always in crystal of the same form and color. But it was afterward demonstrated that hæmatoidin (the coloring matter of the yolk of the egg and corpora lutea) is throughout different from bilirubin of the bile. The orange-red coloring matter of blood-extravasations consists not merely of "hæmatoidin," but also of bilirubin, so that it does not appear improper to call the pigment identical with the coloring matter of the corpora lutea not hæmatoidin, but lutein.
>
> HOLM and STÄEDELER (*Journ. f. pract. Chemie*, 1867, C., p. 142) demonstrated that hæmatoidin and bilirubin are entirely distinct. Well-formed crystals of hæmatoidin by reflected light appear beautifully green (like cantharides), those of bilirubin, orange-red. Hæmatoidin dissolves with bisulphide of carbon with a flame-red or, in dilute solutions, with an orange-red color, bilirubin with a golden yellow. The latter enters into combinations in fixed proportions with alkalies, and is soluble in alkalies, the former not; therefore from a solution of the latter in chloroform it may be separated by agitation with caustic alkalies,

which is not true of the former. Bilirubin furnishes with nitric, containing nitrous acid in alcoholic solution, a beautiful play of colors; green, blue, violet, red, yellow (reaction of biliary matters); hæmatoidin, on the other hand, by nitrous acid is colored light-blue, and then becomes either yellow or colorless. Lutein, according to THUDICHUM (*Med. Ctrlbl.*, 1869, No. 1), is also identical with the yellow coloring matter of butter, fat, blood-serum, and many plants (flowers, stamens, seeds).

SALKOWSKY (HOPPE-SEYLER, *Med.-chem. Unters.*, 3 H., p. 436) found, on the other hand, that hæmatoidin from a strumous cyst had all the peculiarities of bilirubin. He concludes that the hæmatoidin (from corpora lutea) investigated by HOLM was not pure, or that there are different kinds of hæmatoidin.

Following the decade of the 1860s, in 1878, *Charles Thomas Kingzett* of the Council of the Institute of Chemistry of Great Britain and Ireland would state authoritatively (*140*):

There is no established connection whatever between bilirubin or other biliary pigments and the colouring matter of blood; it is necessary to state this emphatically on account of the existence of erroneous statements and impressions to the contrary.

And in 1880, *Legg* (*99*) concluded, on the basis of the existing evidence that hematoidin and bilirubin were not identical, but in 1883, *Hermann* (*141*) identified hematoidin with bilirubin. Toward the end of the 19th century, in 1891, *Ewald* summarized the advances in knowledge of the origin of bile pigments (*142*), leaving little doubt of a consensus that they arise from blood corpuscles and that the hematoidin derived from them is the same as bilirubin (*142*):

The following is a summary of our present tolerably satisfactory knowledge concerning the *bile pigments*.

If we shake bile that has been exposed to the air with chloroform, this takes up a green colouring matter, biliverdin. Fresh bile, however, owes it golden yellow colour to bilirubin, which when pure is an amorphous orange yellow powder, forming, by oxidation in the air or other oxidising means, the green biliverdin (formerly called cholepyrrhin or cholephäin). Chemists have produced a series of intermediate states, especially biliprasin and bilifuscin, and studied their spectroscopic relations and their connections with the blood and urine, which we referred to in the first lecture. Two points especially interest us: the derivation of and tests for the bile-colouring matter. At first sight there seems no doubt that bile pigment is derived from the pigment of the blood corpuscles, hæmochromogen. By injection into the circulation of a whole series of substances which dissolve the blood corpuscles and set free the pigment from them, we succeed in producing bile-coloured urine. Among these solvents are salts of the biliary acids, solutions of hæmoglobin, large quantities of water, chloroform, and ether, common salt solution, glycerine, toluylendiamine, arseniuretted hydrogen; and in the same way jaundice occurs after burning and scalding, after poisoning with oxalic acid, pyrogallic acid, naphthol, phosphorus, &c.; finally, *icterus neonatorum* and the jaundice that occurs in paroxysmal hæmoglobinuria are both due to destruction of blood corpuscles. The same solution of blood pigment and formation of bile pigment may occur naturally in old blood extravasations, where, as you know, peculiar crystals (Virchow's hæmatoidin crystals) have been found, first by Virchow, later by Hoppe-Seyler, also in the margin of the placenta and in the fluids of cysts, while their identity with bilirubin has been ascertained by Jaffé. Moreover, this formation of bile pigment or bilirubin crystals has been observed in artificial extravasations of blood (Langhans, Quincke), in blood injected into the abdominal cavity (Cordua), in frog's blood kept free from putrefaction (v. Recklinghausen). On the other hand, Funke and Zenker found the same crystals in old bile residue. Valentiner prepared hæmatoidin crystals from pulverised gallstones, and

> Schwanda succeeded in extracting characteristic crystals from the urine of a case of jaundice. Neumann found bilirubin crystals in the blood of a three-days' old and probably suffocated child.

Thus, in the last decade of the 19th century it could be said regarding bilirubin (*143*): "*Bilirubin* . . . It is identical with Virchow's hæmatoidin." Yet, as if the subject of the identity of hematoidin would never be put to rest, as more chemical knowledge became available in the early 20th century, a new (and in retrospect radical) theory would emerge in which hematoidin was said to be identical to mesoporphyrin, which was thought to be identical to bilirubin (*144*) – a conclusion that would, rather incredibly, leave both hematoidin and bilirubin as reduced forms of a porphyrin (*144*):

> … Haematoporphyrin is found occasionally in the urine (especially after sulphonal poisoning, which produces considerable blood destruction) and is no doubt derived from haematin, set free from hæmoglobin.
> … Haematoporphyrin, on partial reduction with hydriodic acid, yields *mesoporphyrin*:
>
> $$C_{33}H_{38}O_6N_4 + 2H_2 = C_{33}H_{38}O_4N_4 + 2H_2O$$
> Hæmatoporphyrin. Mesoporphyrin.
>
> … Mesoporphyrin is probably identical with a substance described under the name of *haematoidin*, which was discovered by Virchow in 1847 in blood extravasations, and also with *bilirubin*, which is one of the best-known bile-pigments.

Of course at the time the chemical structures of porphyrins and bile pigments were unknown; so, chemical imagination was not constrained to sensible structures.

In 1923, *Fischer* and *Reindel* (*145*) indicated a probable identity of hematoidin with bilirubin based on the likeness of their crystal forms and their similar behavior on coupling with benzenediazonium chloride. Later, *Rich*,[38] who investigated the origin of bile pigments in the 1920s, provided a useful summary on the status of the subject in 1925 (*146*):

> … Virchow could not prove conclusively that the pigment formed under such circumstances is identical with bilirubin, and he therefore gave it the name of "hematoidin." Indeed, even at the present time there are writers who maintain that the "hematoidin" found in hemorrhages is quite different from true bile pigment (100) or at least that there is no proof that the two substances are identical (37). Of course, since we do not yet know the details of the chemical structure of bilirubin itself, we are unable to say with absolute certainty that bilirubin and "hematoidin" are identical; but they have, apparently, the same percentage composition (although, unfortunately, analyses have been made only upon material obtained from echinococcus cysts of the *liver* (22, (84)) and they are so much alike physically and chemically that most workers who have studied them have felt safe in the

[38] *Arnold Rice Rich* was born on March 28, 1893 in Birmingham, Alabama and died on April 17, 1968 in Baltimore, Maryland. He received the Bachelor's degree in biology at the University of Virginia in 1915 and the Dr. med. in 1919 at Johns Hopkins University. He was a pathologist at Johns Hopkins Medical School, and Professor and Chairman of Pathology in 1944. In 1947, he was appointed the third Baxley Professor of Pathology and remained director of the department until his retirement in 1958.

belief that bile pigment itself can be formed from hemoglobin locally in blood extravasations (Jaffe (39), Quincke (74), Stadelman (94), Hooper and Whipple (35), Van den Bergh and Snapper (103), Leschke (51), McNee (64)). The statements which are scattered throughout the literature concerning the failure of "hematoidin" to give a typical Gmelin test, or to form crystals typical of bilirubin, or to possess the solubility characteristics of bilirubin, – statements which have so often in the past disturbed the acceptance of the identity of these pigments (48), can very probably be referred either to the presence of loosely bound impurities in the "hematoidin" examined, or to some change of such a little-understood nature as that which is known to alter the properties of even gall-bladder bilirubin on standing (1). Rich and Bumstead (80) have subjected "hematoidin," obtained from old hemorrhages, to the long series of physical and chemical tests and reactions which are well established as characteristic of bilirubin, and in every instance the "hematoidin" behaved precisely as did a control of pure bilirubin. In this study it was found that "hematoidin" yields oxidation and reduction products (bilicyanin and urobilin (hydrobilirubin)) which have the same properties and are identical spectroscopically with the substances obtained by the same methods from pure bilirubin. It seems clear that in "hematoidin" we have to deal with a substance which is so much like bilirubin that it cannot be distinguished from the latter pigment by any of our present physical or chemical tests. The burden of proof must, therefore, rest upon those who may deny that true bile pigment can be formed at the site of blood extravasations.[3]

Experimental evidence of the origin of bile pigment from hemoglobin began to appear about 10 years after Virchow's discovery. Interest in the matter was precipitated by the experiments of Frerichs and Städeler (23) who found that a pigment resembling bilirubin could be produced *in vitro* by the action of sulphuric acid upon bile acids,[4] and, more important, that the injection of bile acids into the blood stream of an animal would be followed by the appearance of undoubted bilirubin in the urine. Their conclusion was that the body could transform bile acids into bile pigment. Kühne (44), shortly after, repeated and confirmed a forgotten or unnoticed observation of von Dusch (107) that bile acids are powerful hemolytic agents; and he insisted that experiments of Frerichs and Städeler did not prove the origin of bilirubin from bile acids, for those investigators had not taken into account the fact that a large amount of hemoglobin is set free in the plasma by the injection of bile acids. Kühne was unable to satisfy himself that the injection of hemoglobin alone, in the absence of bile acids, would be followed by bilirubinuria, and he was forced to hold to the idea that the bile acids were necessary in some way for the formation of bile pigment. Herrmann (31), however, in 1859, was able to produce bilirubinuria at will by inducing intravascular hemolysis with injections of distilled water. This was the first clear demonstration that the simple liberation of hemoglobin into the blood stream may be followed by an increased output of bile pigment in the urine. Neither Naunyn (70) nor Steiner (96) could confirm Herrmann's results, and they opposed the conclusion that hemoglobin can be changed by the body into bile pigment. Their failure, as well as that of Kühne, is less difficult to understand now, for we have learned that the appearance of bilirubin in the urine after intravascular hemolysis depends upon a number of factors, and that the absence of bilirubinuria as determined by the Gmelin test, is by no means a proof

[3] In birds in which biliverdin is the predominant pigment of the gall-bladder bile, biliverdin (*i.e.* a bright green pigment which gives a positive Gmelin test) is formed in blood extravasations as well as "hematoidin." This is a further proof of the local formation of true bile pigment in hemorrhages.

[4] Hoppe-Seyler (36) was able to show that this pigment did not really have the properties of bilirubin, and later Städeler (93) himself denied the identity of the two pigments.

that there has been no increased formation of the pigment. Tarchanoff (101), on the other hand, not only confirmed Herrmann's work but, with the use of bile-fistula animals, carried the proof of the relation of hemoglobin to bile pigment still further by demonstrating, for the first time, that the introduction of pure hemoglobin into the circulation is followed by a marked increase in the amount of bile pigment excreted by the liver. Stadelmann (95) confirmed this observation of Tarchanoff in a more carefully controlled series of experiments, and it has since been established beyond question by numerous other investigators, using a variety of experimental animals and procedures, that the liberation of hemoglobin into the blood stream of an intact animal is regularly followed by an increased production of bile pigment which, according to conditions, may be eliminated by the liver or the kidneys, or partially retained in the plasma and tissues producing jaundice (Minowski and Naunyn (66), Gilbert, Chabrol and Bernard (25), Brugsch and Yoshimoto (14), McNee (63), Whipple and Hooper (115), van den Bergh and Snapper (103), and Rich (76)).

The *clinical* evidence of the relation of hemoglobin to bile pigment is to be found in the many different pathological states in which the condition of the liberation of an excessive amount of hemoglobin into the circulation is reproduced. In all of these maladies it is the rule that the formation of bile pigment is increased above the normal level and the pigment content of the feces, the urine and even of the plasma and tissues may be very high.

This was a time, as we shall learn, that the chemical structures of hematoidin, hematin, and bilirubin were still unknown, though it was believed that bilirubin and hemoglobin were closely related chemically, and *Rich*, being unable to find any differences in the physical and chemical properties of hematoidin and bilirubin, thus found them indistinguishable based on the state of knowledge of the times. Yet he was still reluctant to conclude with certainty that they are identical. That would come later, as their chemical structures were revealed.

2.9 Bile Pigment Isolation, Purification, and Combustion Analysis in the 1860s and 1880s

The seventh decade of the 19th century brought about a serious attempt to resolve chaotic differences among chemists regarding atomic weights (relative atomic masses) and equivalents, radicals and molecules, and nomenclature, which resulted in the formula of as simple a molecule as water to be expressed variously as HO, H_2O, and HΘ. Key to a firmer understanding of organic and natural products chemistry was knowing atomic and equivalent weights of which, confusingly, there were, for example three in common use, reflecting disagreements regarding differing atomic weights (relative atomic masses) of carbon and oxygen: those of *Berzelius* (H = 1, C = 12, O = 16), *Liebig* (H = 1, C = 6, O = 8), and *Dumas* (H = 1, C = 6, O = 16), as well as *Gmelin's* system of "equivalents" (H = 1, C = 6, O = 8, N = 14) – each of which had its adherents and practitioners. Thus a formula as simple as ethyl alcohol could be expressed as C_2H_6O (*Berzelius*), $C_4H_{10}O$, H_2O (*Liebig*), or C_8H_8, H_4O_2 (*Dumas*), rendering molecular weight calculations uncertain and limited to guesswork. Yet, by the middle of the decade, the discrepancies had apparently been

resolved by non-unanimous agreement among the 140 participants of the famous Karlsruhe Congress of 1864 (*22*, *147–149*). However, not all of the participating scientists agreed to follow the resolution and adopt the currently accepted atomic weights, which were thus only slowly put into practice, leaving a somewhat confusing array of combustion analyses-derived formulas that were often at variance with each other for the same compound.

At the time, bile pigment isolation had evolved from tedious, imperfect separations of the coloring matter of bile, gallstones and icteric urine. Thus, from the methodology involving repeated precipitations and washings pioneered by *Berzelius* (*68–76*) and his contemporaries in the first half of the century, a new and more efficient method involving $CHCl_3$ extraction was introduced by *Valentiner* (*107*, *108*) and *Brücke* (*109*). The work of the latter two investigators, published in 1858–1859, opened the door to isolation of purified samples of bilirubin and biliverdin, a necessary first step to eventual full characterization of their chemical structure by what was probably the most important analytical method available: combustion analysis. Elemental combustion analysis was developed and applied to organic structure early in the 19th century and by 1860 had evolved into a reliable and effective method of characterization by providing an empirical formula. However, as in most analytical methods, the efficacy of a combustion analysis depends especially, aside from proper analytical technique, on the purity of the sample being analyzed. Up to 1859 and the time of *Brücke*'s work (*109*), the bile pigment samples that had been investigated, whether by combustion analysis or the sensitive, qualitative *Gmelin* test, were of uncertain purity – or of certain impurity. They often contained non-combustible inorganic material, which showed up as residual ash following combustion. And considering the method of isolation, and lacking anything approaching chromatographic methods, it could not be certain that the bile pigment had been freed from other combustible material. As a consequence, though the measured %C, H, and N more often than not had to be adjusted (imperfectly) for ash left behind in the combustion, there was no way to adjust the measurement for organic impurities, rendering any so-derived empirical formulas tenuous at best. A major challenge to the utility of this technique in the second half of the 19th century, and into the 20th, was thus to achieve sample purity, as it often is today.

By 1860, most of the principal investigators of bile pigment "chemistry" had passed on, either permanently or to new endeavors, leaving the two decades between 1860 and 1880 to mainly three individuals, whose names came into prominence in connection with bile pigment analysis: *Städeler*, who published in 1856 with *Frerichs* on the origin of hematoidin from bile acids (*129*); *Thudichum*, who wrote extensively from 1862 to 1881 on gallstones (*101–103*); and *Maly*, who initiated his work with the thesis that *Cholepyrrhin* is the amide of biliverdin (*150*) but soon after adopted great care in experimentation. These

individuals, especially *Städeler* and *Thudichum*, who exhibited more modern laboratory skills and great experimental care coupled with considerable scientific introspection, joined the ranks of the most cited investigators of bile pigment chemistry.

2.9.1 Georg Andreas Karl Städeler Gives the Name Bilirubin as a First Step Is Taken Toward Structure Identification

Städeler's[39] interests and expertise tended more toward chemistry, and early on he became engaged in performing elemental combustion analyses to help develop his theories on the conversion of tyrosine, *inter alia*, to pigments. He also achieved one of the earlier combustion analyses of *Cholepyrrhin*, which he had isolated from bile and purified by the *Valentiner-Brücke* $CHCl_3$ extraction technique (*107–109*). Concerning the first, in 1860, while writing on tyrosine and its reactions (*151*), he noted the formation of a lemon-yellow color following treatment of tyrosine with nitric acid. The color was due to the presence of a red-orange pigment, and what *Städeler* called *dinitrotyrosine* crystallized in golden-yellow blades whose lead salt was colored a chromic-acid red. The red pigment, which had been so easily obtained by oxidation of tyrosine with an excess of HNO_3, he tentatively reserved the name *Erythrosin*. The reddish color apparently reminded him of *Hämatoidin* (hematoidin). *Erythrosin* turned greenish in light and underwent various other color changes upon manipulation with acids and bases. *Städeler* thus noted many similarities between *Erythrosin* and hematoidin and wondered whether a relationship existed between them. Though *Robin's* analysis (*115*, *116*) of hematoidin (64.12% C, 6.87% H, 10.69% N, 18.32% O), from which he gave the formula $C_{14}H_9NO_3$, did not correspond in any way, a re-analysis of hematoidin yielded a satisfactory correspondence to $C_{30}H_{18}N_2O_6$, as comes from the following composition (*129*):

[39] *Georg Andreas Karl Städeler* was born in 1821 in Hannover on 25 March (an historically significant date for the author and for Greek independence), received the Dr. phil. in 1849 and became *Habilitand* at the Universität Göttingen as *Privatdozent* and the first director of *Rudolph Wagner*'s newly established Laboratory for Physiological Chemistry. He was appointed a. o. Professor in Göttingen and, failing to achieve an academic chair (o. Professor) in Breslau, he (and not *Kekulé*, who was also interested) was appointed to o. Professor at the University of Zürich, where he more fully engaged in his academic career until his death in Hannover on 11 January 1871. Among his colleagues in Göttingen, he found *Friedrich Frerichs*, who was nearly the same age and with whom he struck up a close friendship. *Frerichs* was *Assistent* in *Rudolf Wagner*'s Laboratorium für physiologische und pathologische Chemie from 1843 to 1850 before he moved to Kiel as a.o. Professor für Pathologie and Vorstand der Poliklinik. *Städeler*'s name was linked to *Frerichs*' through their jointly published work on the conversion of certain amino acids to pigments (*127*, *128*) and, in animal metabolism, the conversion of intravenously-injected bile acids into bile pigments found soon afterward in urine (*129*).

				Berechnet[1]		Gefunden	
30	Aeq.	Kohlenstoff	[C]	180	65,69	65,85	65,05
18	„	Wasserstoff	[H]	18	6,57	6,47	6,37
2	„	Stickstoff	[N]	28	10,22	10,50	10,50
6	„	Sauerstoff	[O]	48	17,52	17,18	18,08
				274	100,00	100,00	100,00

Using his new formula for hematoidin, *Städeler* then wrote a chemical equation showing how oxidation of tyrosine, coming from decomposition of proteins in organisms, might produce hematoidin (*129*):

> Nimmt man diese Formel für das Hämatoïdin an, so würde sich dieselbe vom Tyrosin ableiten lassen:

$$\underbrace{2\,C_{18}H_{11}NO_6}_{\text{Tyrosin}} + 2\,O = \underbrace{C_2O_4}_{\text{Kohlensäure}} + \underbrace{C_4H_4O_4}_{\text{Essigsäure}} + \underbrace{C_{30}H_{18}N_2O_6}_{\text{Hämatoïdin}}$$

> Der Grundfarbstoff des Bluts, das Hämatoïdin, könnte also durch einen Oxydationsprocess aus dem Tyrosin, das beim Zerfall der Proteïnstoffe im Organismus ensteht, hervorgehen. 70

Of course, the formulas above are based on the atomic weights C = 6, H = 1, N = 14, O = 8, which were revised in the Karlsruhe Conference of 1864 (*22*, *147–149*), but not widely or immediately accepted. As in the calculation of an empirical formula, this point too illustrates the state of chemistry and how easy it is fall into a trap in the absence of more correct information. *Städeler* did not assert, however, that hematoidin and *Erythrosin* were identical. He only proposed the possibility and indicated that he would pursue the question as soon as his time permitted:

> Damit soll übrigens keineswegs behauptet werden, dass Hämatoïdin und Erythrosin identisch seien; für möglich halte ich diess allerdings und ich werde daher, sobald es meine Zeit erlaubt, die Frage weiter verfolgen. 71

Städeler's analysis of *Cholepyrrhin* was reported in a footnote in volume II of *Frerichs*' *Klinik der Leberkrankheiten* published in 1861 and translated by *Murchison* in the same year (*120*):

> The elementary analysis performed by my friend Professor Städeler, of Zurich of cholepyrrhin purified by repeated crystallization from boiling, and washing with cold chloroform, yielded results from which the chemical formula $C_{18}H_9NO_4$ is calculated.

[1] N.B. *Berechnet* = Calculated; *Gefunden* = Found. It may be noted that the calculations above are based on *Gmelin*'s system of "equivalents", where atomic mass carbon = 6, hydrogen = 1, nitrogen = 14, and oxygen = 8. Had the current atomic weights been used, the empirical formula would be $C_{15}H_{17}NO_3$.

					Calculated formula.		Actual Result of Analysis.
18	equivalents	of	carbon	=	108	66·26	66·52
9	„	„	hydrogen	=	9	5·52	6·00
1	„	„	nitrogen	=	14	8·59	8·70
4	„	„	oxygen	=	32	19·63	18·78
					163	100·00	100·00

Hence cholepyrrhin only differs from isatine, the product of the oxydation of indigo, by the elements of one equivalent of hyduret of methyle.

$$\left.\begin{matrix} C_{16}H_5NO_4 + C_2H_3 \\ H \end{matrix}\right\} = C_{18}H_9NO_4$$

Moreover, cholepyrrhin contains 2 equivalents of water less than tyrosine, and 2 equivalents of oxygen less than hippuric acid. According to this, the occurrence of indigo in human urine, which has been repeatedly observed, is a less remarkable circumstance than might at first be thought. It will be interesting to study more closely the relations between cholepyrrhin and isatine.

From these data, with no reported residue of ash, *Städeler* derived the chemical formula $C_{18}H_9NO_4$, which is somewhat different from his analytical data that predicted $C_{30}H_{18}N_2O_6$ for hematoidin (*151*), and also that from *Robin* (*115*, *116*), thus *Städeler* wrote (*151*): $C_{14}H_9NO_3$.[40]

With an apparently more direct focus on *Cholepyrrhin*, in 1864, *Städeler* published what might be considered a landmark paper (*135*), this nearly four years subsequent to his earlier reported combustion analysis. Also, in 1864, *Maly* published his preliminary studies (*150*), and only a year earlier *Thudichum* had published his important work on the same pigment (*102*, *103*). *Städeler* comprehensive work (*135*) briefly reviewed previous studies of others on the pigments of bile, and then related his own studies on the pigments from bile and gallstones. From the perspective of the mid-19th century, this publication is an impressive scientific endeavor and a tribute to *Städeler*'s clarity of thought and attention to detail. The work presented represents an elevation in knowledge and thought regarding bile pigments.

Yet perhaps *Städeler*'s longest lasting contribution to bile pigments was the name *Bilirubin* (*Gallenroth*, red-bile = red pigment of bile; Latin: *bilis*, bile; *rubris*, reddish) that he gave to the purified reddish pigment of bile and gallstones. The name apparently caught on, was adopted increasingly widely, and has been accepted for more than 100 years as the standard name of the pigment. In fact, the name was apparently so appealing and logical that even *Thudichum*, who coined his own

[40] N.B. *Städeler* was consistent in his use of *Gmelin*'s system of atomic equivalents rather the actual atomic values agreed upon at the 1860 Karlsruhe Conference. A recalculation of the data using the atomic weights of today would give the empirical formula $C_{18}H_{19}N_2O_4$ for *Cholepyrrhin* and $C_{15}H_{17}NO_3$ for hematoidin, but even these are not the correct values. Those were learned only decades later.

words for the bile pigments, adopted it before 1868 (*152*), and it began to appear in medical and physiology textbooks within a decade or so of *Städeler*'s introducing it. Thus, one may find the name *bilirubin* in various subsequent authoritative sources, such as: *Pflüger*'s 1871 *Archiv für die gesammte Physiologie des Menschen und der Thiere* (*153*), *Wood*'s *Report on Medical Chemistry* in 1873 in *The Boston Medical and Surgical Journal* (*154*), *Wagner*'s 1876 *A Manual of General Pathology* (*99*), *Kingzett*'s 1878 *Animal Chemistry* (*140*), *Legg*'s 1880 *On the Bile, Jaundice and Bilious Diseases* (*99*), and in later publications.

Städeler reviewed what were the then most recent combustion analyses from *Scherer*, *Hein*, and *Heintz*, especially those obtained by *Heintz* some 13 years earlier (*97*) for *Biliphäin* and biliverdin. The *Biliphäin* analyzed was suspected to be a mixture of pigments, and *Städeler* believed that *Valentiner*'s (*107*, *108*) successful $CHCl_3$ extraction of *Gallenroth* from bile and gallstones proved it. He also thought that *Brücke* (*109*) proved that *Biliphäin* is converted to biliverdin by absorption of oxygen. Since *Valentiner*, too, took *Gallenroth* to be identical to hematoidin, *Städeler* was puzzled by *Robin*'s formula for hematoidin (based on its combustion analysis) because he believed that if it were correct then biliverdin could not arise from oxidation of hematoidin (*135*):

> Eine Analyse des Gallenrothes ist nicht gemacht worden, und vergleicht man die Formel, welche sich aus Robin's Analysen für das *Hämatoïdin* . . . berechnet: $C_{30}H_{18}N_2O_6$ mit der Formel des *Biliverdins*: $C_{16}H_9NO_5$ oder $C_{32}H_{18}N_2O_{10}$, so ergiebt sich, dass das letztere im Verhältniss zum Stickstoff mehr Kohlenstoff enthält, als das Hämatoïdin, dass also, wenn Robin's Analysen richtig sind, das Biliverdin nicht durch Oxydation aus dem Hämatoïdin entstehen kann. 72

His interest in bile pigments was also driven by his work with *Frerichs* (*129*), and that of *Neukomm* (*130*) in his lab in Zürich, on the production of bile pigments from bile acids, for which he proposed two explanations: (i) intravenously injected bile acids are convered directly into bile pigments in the bloodstream, or (ii) bile acids influence bile pigment production from hemoglobin or hematin. In order to pursue a comparative chemical investigation of synthetic and naturally occurring bile pigments, he took up an investigation of the latter (*135*):

> Meinungsverschiedenheiten herrschen nur darüber, ob die Gallensäuren in der Blutbahn direct in Pigmente verwandelt werden, oder ob die Pigmentbildung der auflösenden Wirkung dieser Säuren auf das Blutroth zugeschrieben werden müsse. Durch blosse Injectionsversuche, wie es bisher geschehen ist, liess sich die Frage offenbar nicht genügend beantworten, während von einer vergleichenden chemischen Untersuchung der künstlichen und der natürlich vorkommenden Gallenpigmente bestimmte Aufschlüsse zu erwarten standen.
>
> Um diese Vergleichung vornehmen zu können, habe ich mich zunächst mit einer Untersuchung der natürlichen Gallenpigmente beschäftigt. – Indem ich die erhaltenen Resultate mittheile, benutze ich zugleich die Gelegenheit, allen Freunden und Collegen, die mich durch Zusendung von Material bei dieser Untersuchung unterstützt haben, meinen Dank hiermit auszusprechen. 73

In order to obtain the natural pigment(s), he turned to pigmented gallstones as a source of bilirubin by processing according to *Valentiner*'s method. After removing fats and cholesterol from the pulverized stones, followed by a hot water wash to

remove traces of bile, he extracted with $CHCl_3$ to obtain a small amount of sticky, greenish-brown residue (after evaporation) that contained *Gallenroth* crystals, as seen under a microscope. The powdered gallstone residue, after the $CHCl_3$ extraction, was treated with dilute HCl to dissolve a large quantity of calcium and magnesium salts and evolve CO_2. The resulting dark brown residue, after washing and drying, yielded a large amount of pigment into boiling $CHCl_3$, thus suggesting to *Städeler* that the majority of the pigment had been originally bound up as salts. Evaporation of this $CHCl_3$ extract gave a dark solid-crystalline residue, from which a "brown pigment (among other material)" was extracted into hot alcohol. *Städeler* named it *Bilifuscin* (Latin: *bilis*, bile; *fuscus*, dark). The gallstone residue, after the boiling $CHCl_3$ extraction above, contained a considerable amount of *Gallenroth* (bilirubin), albeit in impure condition. After as much "brown pigment" as possible had been extracted with $CHCl_3$, the solid residue was colored bright olive and still contained considerable *Gallenroth* as well as a green pigment that *Städeler* called *Biliprasin* (from Latin: *bilis*, bile; *prasinus*, green), which was washed exhaustively with alcohol to give a beautiful green colored solution. Then the remaining *Gallenroth* was extracted into boiling $CHCl_3$. The residue, after all of the washings/extractions, was insoluble in H_2O, alcohol, ether, $CHCl_3$, and dilute acids. It reminded *Städeler* of *humin* (soil), and thus he found the name *Bilihumin* (Latin: *bilis*, bile; *humis*, soil) appropriate.

Essentially following *Brücke*'s method (*109*), *Städeler* further purified the $CHCl_3$-extracted *Gallenroth*, taking it through several cycles of dissolving it in $CHCl_3$, filtering, and evaporating, washing the residue each time with ether and alcohol. The alcohol washings were always more or less green to greenish-brown, while the bilirubin remained as a vivid red to orange-red granular-crystalline powder. With this purified bilirubin, *Städeler* proceeded to its combustion analysis, from which he discovered that the data corresponded to no acceptable formula. Just what constituted an acceptable formula is unclear. In an analysis mentioned in 1861 by *Frerichs* (*120*), *Städeler* had found $C_{18}H_9NO_4$ from 66.52% C, 6.00% H and 8.70% N, which was later found to be unacceptable. In any event, *Städeler* repurified his bilirubin by precipitating it with alcohol from a $CHCl_3$ solution (*135*):

> 1) *Bilirubin*. – Um diesen Farbstoff, der in vorwiegender Menge in den menschlichen Gallensteinen vorkommt, zu reinigen, wurde er einige Male in Chloroform gelöst, die filtrirte Lösung verdunstet und der Rückstand mit Aether und Weingeist gewaschen. Der abfliessende Weingeist zeigt sich immer mehr oder minder grün bis grünlichbraun gefärbt, während das Bilirubin als ein lebhaft rothes bis orangerothes, körnig-krystallinisches Pulver zurückblieb.
>
> Bei der Analyse des so gereinigten Farbstoffes wurden Zahlen erhalten, die mit keiner annehmbaren Formel genügend übereinstimmen, woraus auf eine Verunreinigung geschlossen werden musste. Diese zu beseitigen gelang mir dadurch, dass ich die Chloroformlösung nur bis zur beginnenden Abscheidung von Bilirubin verdunsten liess und sie dann durch Zusatz von Weingeist fällte. Auf diese Weise wurde das Bilirubin als amorphes orangefarbenes Pulver erhalten; ein ziemlich bedeutender Verlust war dabei nicht zu vermeiden. 74

A similar procedure is still used today to purify the commercially available pigment (*13*).

Significantly, the purified pigment left no ash upon combustion and was dried at 100°C over conc. H_2SO_4 to lose 1% of its weight. Further heating between 120°C and 130°C produced no further reduction in weight. The material was thus deemed suitable for combustion analysis, but before it was accomplished, however, *Städeler* observed that by heating it in a glass (melting point) tube, the solid became swollen and evolved a yellow, foul-smelling vapor that blackened lead paper – a test typically used to detect H_2S. (It is unclear how bilirubin, which contains no sulfur, might evolve H_2S upon heating.) Nonetheless, the trace of sulfur was thusly shown to be present in all the pigments of *Städeler*'s study. It did not come from sulfate, as combustion of the bilirubin with lime (CaO) and salt-peter (niter or any nitrate, usually KNO_3) followed by acidification of the residue with HCl produced no turbidity upon addition of $BaCl_2$ (*135*):

> Der erhaltene Farbstoff verbrannte auf Platinblech, ohne einen Rückstand zu hinterlassen. Nach mehrtägigem Stehen über Schwefelsäure verlor er bei 100º nahezu 1 pC. an Gewicht. Bei weiterem Erhitzen auf 120 bis 130º blieb das Gewicht constant. Beim Erhitzen im Glasrohr schmolz das Bilirubin, es blähte sich auf und entwickelte gelbe übelriechende Dämpf, welche Bleipapier schwärzten. Dagegen wurde beim Verbrennen von 0,176 Grm. Substanz mit Kalk und Salpeter, Auflösen der geglühten Masse in verdünnter Salzsäure und Zusatz von Chlorbaryum keine Trübung wahrgenommen. – Die durch Bleipapier angezeigte Spur von Schwefel war auch in allen übrigen Pigmenten der Gallensteine nachzuweisen.
>
> Das zu den folgenden Analysen benutzte Bilirubin war bei zwei Darstellungen erhalten worden.
>
> I. 0,3765 Grm., bei 120º getrocknet, gaben 0,927 Grm. Kohlensäure und 0,2125 Grm. Wasser.
> 0,2563 Grm, bei derselben Temperatur getrocknet, lieferten bei der Verbrennung mit Natronkalk eine Quantität Salmiak, aus welcher mit salpetersaurem Silber 0,252 Grm. Chlorsilber gefällt wurden.
>
> II. 0,3105 Grm., bei 130º getrocknet, gaben 0,764 Grm. Kohlensäure und 0,171 Grm. Wasser.
> Aus diesen Daten berechnet sich für das Bilirubin die Formel $C_{32}H_{18}N_2O_6$.[41]

			berechnet		I.	II.
32	Aeq	Kohlenstoff	192	67,13	67,15	67,11
18	„	Wasserstoff	18	6,29	6,27	6,12
2	„	Stickstoff	28	9,79	9,59	–
6	„	Sauerstoff	48	16,79	16,99	–
			286	100,00	100,00	

75

The properties of this highly purified bilirubin are described by *Städeler* in some detail, properties that anyone today might recognize as characteristic of this pigment: The pigment was orange-colored in the amorphous state (colored somewhat like Sb_2S_3); in the crystalline state, the crystals were well-formed and measurable, with the vivid dark color of chromic acid. It was insoluble in ether, soluble in traces

[41] N.B. The recalculated formula using conventional atomic weights would be $C_{16}H_{19}N_2O_3$.

in ethanol but dissolved in cold $CHCl_3$ to give a yellow to yellow-orange solution. The more crystalline it was, the more difficult it was to effect dissolution in $CHCl_3$; continuous heating was required. And it was noted that pure $CHCl_3$ became rapidly acidic and generated phosgene. In such $CHCl_3$ the erstwhile yellow color turned to green. But when the $CHCl_3$ contained a bit of ethanol, there was no color change. (And so commercial $CHCl_3$ typically contains a little ethanol as stabilizer). Benzene and CS_2 were good solvents; turpentine and fatty oils (almond oil) dissolved the pigment upon warming and produced a yellow color. It dissolved in alkali giving deep orange solutions that became yellow at high dilution. The dilution experiments are interesting. With a 15 mm thick layer of the alkaline (NH_4OH) solution as the standard reference, a dilution factor of 15,000 still left an orange color; a 20,000 dilution factor left a deep golden yellow; from a factor 25,000–100,000 it was pure yellow, as in solutions of neutral K_2CrO_4. At a factor of 5×10^5, and at 10^6 at twice the sample thickness the yellow color was still noticeable. Dilutions of 3–4 $\times 10^4$ imparted a distinctly yellow coloration to the skin. Thus such extraordinary tinctural power easily explained the yellow coloration of skin and eyes at the occasional rapid onset of jaundice. From the coloration of the eyes due to intense icterus, one might conclude an approximate 2–2.5 $\times 10^4$ dilution of the pigment. From this, *Städeler* appeared to imply a visual method for diagnosing the severity of jaundice (*135*):

> 30- bis 40000 fach verdünnte Lösungen färben die Haut noch deutlich gelb. – Bei so ausserordentlichem Farbvermögen ist das mitunter so rasche Eintreten von Gelbsucht, die gelbe Färbung des Auges und der Haut, leicht erklärlich. Aus der Farbe des Auges bei intensivem Icterus darf man auf etwa 20– bis 25000 fache Verdünnung des Pigmentes schliessen. 76

The alkaline ammonia solutions above were found to bleach, even if not completely, moderately rapidly in direct sunlight, while in diffuse light bleaching occurred only slowly. The solutions gradually became light brownish-yellow and lost the ability to be precipitated upon addition of hydrochloric acid, while from the undecomposed solution, even at great dilution, bilirubin precipitated at once in orange colored flakes upon addition of the HCl. Apparently *Städeler* was the first to record a photooxidation or photooxygenation reaction of bilirubin, one assumes in the presence of air – thereby anticipating the early photochemical investigations (see Chapter 9) of the molecular mechanisms of phototherapy for neonatal jaundice (*155–157*). Thus, from *Städeler* (*135*):

> Die mitgetheilten Bestimmungen der Farbenintensität wurden mit ammoniakalischen Bilirubinlösungen gemacht; solche Lösungen bleichen, wenn auch nicht vollständig, ziemlich rasch im directen Sonnenlicht, während sie sich im zerstreuten Licht nur langsam zersetzen. Sie werden allmälig hellbräunlich gelb und verlieren die Eigenschaft durch Salzsäure gefällt zu werden, während sich aus der unzersetzten Lösung, auch bei grosser Verdünnung, auf Zusatz von Salzsäure sogleich Bilirubin in orangefarbigen Flocken abscheidet. 77

Städeler noted some differences between solutions of bilirubin in aqueous ammonia, NaOH, and Na_2CO_3, and that these aqueous basic solutions extract all of the pigment from its solution in $CHCl_3$. He indicated that compounds of bilirubin

with "earths" and heavy metal oxides were insoluble or barely soluble in H_2O. A voluminous rust-colored calcium compound was precipitated by addition of $CaCl_2$ to an aqueous ammonia solution of the pigment. The dried compound was a splendid dark green, with a metallic reflection. Pulverizing it yielded a dark brown powder of the color of pigment-rich human gallstones that to the greatest part also consisted of this compound. The calcium compound was as good as insoluble in ether, alcohol, and $CHCl_3$, and when heated in the last two solvents gave only a weakly yellow color. In a similar way, the salts with $BaCl_2$, sugar of lead, $Pb(OAc)_2$, and $AgNO_3$ produced barium, lead, and silver compounds. The last precipitated in brownish-violet flakes that could be heated without reduction of the silver. The calcium compound analyzed for $C_{32}H_{17}N_2O_6Ca$ (*135*):

> 0,2549 Grm. hinterliessen beim Verbrennen, Anfeuchten der Asche mit kohlensaurem Ammoniak und Trocknen bei 130º 0,0414 Grm. kohlensauren Kalk, übereinstimmend mit der Formel: $C_{32}H_{17}CaN_2O_6$. Die Rechnung verlangt 9,18 pC. Kalk; gefunden wurden 9,10 pC. 78

Städeler treated bilirubin systematically with HNO_3, producing new results and a calibration of the *Gmelin* reaction. Warming bilirubin with dilute HNO_3 (20% H_2O) produced dark violet resinous flakes that became light brownish with further heating and dissolved to form a yellow solution. In the cold there was essentially no change, but with more dilute HNO_3 (30% H_2O) bilirubin formed resinous flakes in the cold and became reddish colored; upon heating the mixture ended up as a yellow solution, as above. If pure HNO_3 hydrate was used, bilirubin dissolved immediately in the cold with a dark red color, and after a little while, or by heating, the solution lightened but retained a bright cherry-red color upon standing after several days (*135*):

> Uebergiesst man Bilirubin mit einer verdünnten Salpetersäure, welche 20 pC. Hydrat enthält, so bemerket man in der Kälte keine wesentliche Einwirkung; beim Erwärmen damit verwandelt es sich dagegen in dunkelviolette Harzflocken, die bei weiterer Einwirkung hellbräunlich werden und sich beim Aufkochen mit gelber Farbe lösen. Eine Säure mit 30 pC. Hydrat bildet die Harzflocken schon in der Kälte und färbt sich röthlich; beim Erwärmen verschwinden die Flocken und die Lösung wird gelb. Wendet man reines Salpetersäurehydrat an, so löst sich das Bilirubin schon in der Kälte mit tief rother Farbe, und nach einiger Zeit oder beim Erhitzen wird die Lösung heller, behält aber selbst bei mehrtägigem Stehen eine lebhaft kirschrothe Farbe. 79

If bilirubin was dissolved in commercial conc. HNO_3, to which one added a little fuming red acid, the well-known bile pigment reaction (*Gmelin* reaction) was thus seen outstandingly. It was best to use alkaline solutions before the addition of HNO_3 and mix them with an approximately equal volume of alcohol. Upon addition of HNO_3 a magnificent reaction was seen even when the added acid contains no nitrous acid, and the sample was not turbid with precipitated flakes of pigment. As *Gmelin* observed decades earlier, the yellow color goes green first, then blue, violet, ruby red, and finally dirty yellow. By not stirring, all of the colors could be seen at the same time, as layer upon layer. The limits of detection were excellent: 0.25 mg bilirubin in a 4 cm^3 solution still produced a splendid display of colors. The entire reaction occurred best at a dilution factor of $7–8 \times 10^5$ (*135*):

> Vermischt man Lösungen des Bilirubins mit käuflicher concentrirter Salpetersäure, der man zweckmässig etwas rothe rauchende Säuer zusetzt, so erhält man die bekannte

> Gallenpigmentreaction in ausgezeichnetem Grade. Am besten wendet man alkalische Lösungen an und vermischt dieselber vor dem Säurezusatz mit ungefähr dem gleichen Volumen Weingeist. Bei Weingeistzusatz erhält man eine prachtvolle Reaction auch dann, wenn die anzuwendende Säure keine Untersalpetersäure enthält, und die Probe wird durch ausgeschiedene Pigmentflocken nicht getrübt. Die gelbe Farbe geht zuerst in grün über, wird dann blau, violett, rubinroth und endlich schmutzig gelb. Wird nicht geschüttelt, so zeigen sich alle diese Farben gleichzeitig schichtenweise über einander. ¼ Milligr. Bilirubin in 4 CC. Lösung bringt noch ein prächtiges Farbenspiel hervor. Die Grenze der Reaction tritt erst bein 70– bis 80000 facher Verdünnung ein. 80

The blue pigment formed fleetingly in the *Gmelin* reaction was of interest to *Städeler* in connection with suspected indigo in urine. Why indigo might be present is anyone's guess, but *Städeler* isolated the blue pigment from bilirubin without difficulty. He did this essentially by dropwise addition of the acid mixture (above) to a "not too dilute" solution of bilirubin in aqueous ammonia, and eliminated too great an excess of HNO_3 by neutralizing with ammonia. All this produced at first a green flocculent precipitate that gradually became blue. After washing the precipitate with H_2O, the co-mixed green pigment was removed with alcohol to leave behind a dark blackish-blue powder. The likely view is that this blue pigment was related to the indigo content of urine. *Städeler* expressed the misfortune of not having sufficient material to be able to undertake further experiments on it.

The blue pigment could also be obtained from a yellow $CHCl_3$ solution of bilirubin by mixing in 1–2 drops of HNO_3 and shaking. This resulted in a very dark liquid that soon went violet, then ruby red. If alcohol were quickly added and mixed in as soon as the violet color appears, the solution became dark blue and changed color only very slowly. Using this approach, a splendid green or red was produced, colors that depend on an earlier or later addition of alcohol (*135*):

> Das bei der angegebenen Reaction entstehende blaue Pigment lässt sich ohne Schwierigkeit isoliren. Vermischt man eine nicht zu verdünnte ammoniakalische Bilirubinlösung tropfenweise mit der oben angegebenen Säuremischung, und beseitigt von Zeit zu Zeit einen zu grossen Ueberschuss von Salpetersäure durch annähernde Neutralisation mit Ammoniak, so erhält man zuerst einen grünen flockigen Niederschlag, der allmälig blau wird. Nach dem Auswaschen mit Wasser kann ihm beigemengtes grünes Pigment durch Weingeist entzogen werden und es bleibt dann ein tief-schwarzblaues Pulver zurück. Die Ansicht liegt nahe, dass dieses blaue Pigment in Beziehung steht zu dem Indiggehalt des Harns. Leider besass ich nich genug Material, um Versuche in dieser Richtung anstellen zu können.
>
> Ein prachtvolles Blau kann man auch bei Anwendung von Chloroform erhalten. Wird eine gelbe Chloroformlösung des Bilirubins mit einem oder zwei Tropfen Salpetersäure vermischt und geschüttelt, so wird die Flüssigkeit sehr dunkel, bald in's Violette übergehend und dann rubinroth werdend. – Setzt man, sobald der violette Farbenten eingetreten ist, rasch viel Weingeist hinzu, so erfolgt Mischung, die Lösung wird tief blau und verändert nur langsam ihre Farbe. – Auf gleiche Weise kann man auch ein prachtvolles Grün oder Roth erzeugen; die Farbe hängt ab von dem früheren oder späteren Weingeistzusatz. 81

Städeler noted that bilirubin dissolved in cold, conc. H_2SO_4 to produce a brownish liquid that gradually turned violet-green. Addition of H_2O separated dark green, nearly black flakes that dissolved in alcohol with a marvellous violet color. Addition of HNO_3 gave a beautiful display of colors, with the red being especially

vivid and beautiful. On the other hand, by heating bilirubin in fuming HCl, the solution became dark brown (*Städeler* thought possibly due to *Bilifuscin* formation). Decomposition appeared to proceed to *Humin* formation, and by heating longer a brown compound resulted that was insoluble in dilute ammonia.

He carried out what might be the first experiments involving reduction of bilirubin by treating a dark red-brown alkaline solution of the pigment with Na(Hg) – a method often used subsequently, even some hundred years later in the *C.J. Watson* lab at the University of Minnesota. The color rapidly decreased, and the solution became pale yellow, a coloration which did not vanish upon warming. *Städeler* was not able to investigate the resulting compound further, which he believed to remain probably in a similar relationship to blirubin as is indigo white to indigo blue. Assuming this is correct, then the (new) yellow pigment would have the composition formula $C_{32}H_{20}N_2O_6$. (Or two more hydrogens than in the blirubin formula given by *Städeler* above) (*135*):

> Reducirende Materien wirken sehr energisch auf das Bilirubin ein. Vermischt man die tief-rothbraune alkalische Lösung des Farbstoffs mit Natriumamalgam, so nimmt die Farbe rasch ab und die Lösung wird blassgelb; auch beim Erwärmen verschwindet dieser Farbenton nicht. Ich habe den hierbei entstehenden Körper, der wahrscheinlich in demselbe Verhältniss zum Bilirubin steht, wie das Indigweiss zum Indigblau, nicht näher untersuchen können. Ist das angedeutet Verhältniss richtig, so würde dieser gelbe Körper der Formel $C_{32}H_{20}N_2O_6$ entsprechend zusammengesetzt sein. 82

Before turning from the pigments of gallstones to the pigments of human bile, *Städeler* addressed biliverdin in his by now evidently comprehensive fashion. To make biliverdin, as was done by others in the past, he oxidized a solution of bilirubin in aq. NaOH using air; after rapid uptake of oxygen the solution turned green. When at its greatest intensity, hydrochloric acid was added to produce a strongly green precipitate that was insoluble in ether and in $CHCl_3$. As *Brücke* noted earlier (*109*), it dissolved in alcohol leaving unreacted bilirubin behind as orange flakes, and the green solution gave a positive *Gmelin* reaction; turning blue, then violet, red, and finally a dirty yellow. *Städeler* was convinced that the green pigment was the same as that which *Heintz* had analyzed by combustion and for which he established the formulas $C_{16}H_9NO_5$, or $C_{32}H_{18}N_2O_{10}$ (*95*). These formulas would be produced from *Städeler*'s bilirubin formula by the addition of four oxygen atoms. Yet from his own analyses, *Städeler* remained doubtful (*135*):

> 2) *Biliverdin*. – Wird eine Lösung von Bilirubin in überschüssiger Natronlauge auf flachen Tellern der Einwirkung der Luft ausgestezt oder anhaltend mit Luft geschüttelt, so nimmt sie ziemlich rasch Sauerstoff auf und die Lösung wird grün. Hat diese Farbe ihre grösste Intensität erreicht, so entsteht auf Zusatz von Salzsäure ein lebhaft grüner Niederschlag, der in Aether und in Chloroform unlöslich ist, während er sich in Weingeist sehr leicht mit prachtvoll grüner Farbe auflöst. Etwa beigementes unzersetztes Bilirubin bleibt dabei in orangefarbenen Flocken zurück. Salpetersäure färbt die grüne Lösung zuerst blau, dann violett, roth und schliesslich schmutzig gelb.
>
> Dieses grüne Pigment ist ohne allen Zweifel das von Heintz . . . analysirte Biliverdin, wofür er die Formel $C_{16}H_9NO_5$ oder $C_{32}H_{18}N_2O_{10}$ aufgestellt hat.

Nimmt man diese Formel als richtig an, so würde die Bildung des Biliverdins aus dem Bilirubin auf einfacher Oxydation beruhen:

$$\underbrace{C_{32}H_{16}N_2O_6}_{\text{Bilirubin}} + 4\,O = \underbrace{C_{32}H_{18}N_2O_{10}}_{\text{Biliverdin}} \qquad 83$$

Aber ich habe einige Beobachtungen gemacht, welche die Richtigkeit dieser Formel bezweifeln lassen.

Air oxidation of bilirubin interested *Städeler*, and following his keen investigative instincts, he found that the pigment dissolved in cold aq. NaOH without change and precipitated in orange-colored flakes with excess added (supersaturated with) HCl. A solution of bilirubin in ammonia behaved likewise and it made no difference therefore whether the solution was prepared cold or had been heated previously. In contrast if an NaOH solution were heated, even with complete absence of air, a remarkable color change was observed. The red solution became dark brown to green-brown and, when supersaturated with hydrochloric acid, a dark green, and not an orange, precipitate was obtained. Treatment of the same with alcohol left a dirty yellow matter on the filter paper, while the pigment, which was found in the splendid green filtrate, possessed all the properties of biliverdin. Its solution in alkalis, especially, was green, by which biliverdin was most easily distinguished from *Biliprasin*, which dissolved in alkalis with a brown color.

Formation of biliverdin simply by heating an aq. NaOH solution of bilirubin seemed to *Städeler* to stand in the way of acceptance of the formula proposed by *Heintz* (*97*). If one compares *Heintz*'s analytical results with his formula a satisfactory correspondence is in no way shown that might compel one to regard the formula as definitely established. The carbon and nitrogen content of the analysis fit better to the formula $C_{32}H_{20}N_2O_{10}$ than to *Heintz*'s $C_{32}H_{18}N_2O_{10}$ formula (*97*), while the hydrogen content found lay in the middle between the two formulas (*135*):

Der gefundene Kohlenstoff- und Stickstoffgehalt stimmt besser mit der Formel $C_{32}H_{20}N_2O_{10}$ überein, während der gefundene Wasserstoff in der Mitte zwischen beiden Formeln liegt:

	$C_{32}H_{20}N_2O_{10}$	gefunden	$C_{32}H_{18}N_2O_{10}$
Kohlenstoff	60,00	60,04	60,38
Wasserstoff	6,25	5,84	5,66
Stickstoff	8,75	8,53	8,80
Sauerstoff	25,00	25,59	25,16
	100,00	100,00	100,00.

Wahrscheinlich was das von Heintz analysirte Biliverdin nicht vollkommen rein, da es aus einem Farbstoffgemenge, aus dem s. g. Biliphäin, durch Auflösen in kohlensaurem Natron und freiwillige Oxydation erhalten wurde. Ich bedaure daher um so mehr, gegenwärtig nicht im Besitze einer genügenden Menge von reinem Bilirubin zu sein, um das Biliverdin einer neuen Analyse unterwerfen zu können. 84

Städeler believed that *Heintz* had analyzed impure biliverdin since it had been obtained from a pigment mixture precursor, the so-called *Biliphäin*, by dissolving in aq. Na_2CO_3 and allowing it to oxidize spontaneously. *Städeler* considered himself

unfortunate not to have had in his possession a sufficient amount of bilirubin to produce biliverdin and undertake a new combustion analysis of it. Given the formula $C_{32}H_{20}N_2O_{10}$ for biliverdin, *Städeler* felt that the pigment stood in relationship to bilirubin as did *Biliprasin* to *Bilifuscin*, and its formation by oxidation of bilirubin would yield the equation (*135*):

$$\underbrace{C_{32}H_{18}N_2O_6}_{\text{Bilirubin}} + 2\,HO + 2\,O = \underbrace{C_{32}H_{20}N_2O_{10}}_{\text{Biliverdin}}{}^{[1]}$$

[1] N.B. These formulas are based on *Gmelin's* system of atomic "equivalents", which for H = 1, C = 6, and O = 8, water becomes HO.

In order to explain his own conversion of bilirubin to biliverdin simply by heating in aq. NaOH, *Städeler* theorized that two equivalents of bilirubin were involved to give one equiv. of biliverdin and one of the same compound that was formed when bilirubin was treated with Na(Hg) (*135*):

$$\underbrace{2\,C_{32}H_{18}N_2O_6}_{\text{Bilirubin}} + 4\,HO = C_{22}H_{20}N_2O_6 + \underbrace{C_{32}H_{20}N_2O_{10}}_{\text{Biliverdin}}$$

Städeler continued to rationalize the formation of the other compounds found in gallstones: (brown) *Bilifuscin*, (green) *Biliprasin*, and *Bilihumin*. He had not found more than traces of biliverdin in gallstones and theorized that it had been converted earlier in bile to *Biliprasin* (*135*):

$$\underbrace{C_{32}H_{20}N_2O_{10}}_{\text{Bilirubin}} + 2\,HO = \underbrace{C_{32}H_{22}N_2O_{12}}_{\text{Biliprasin}}$$

> Ich bemerke noch, dass ich das Biliverdin nicht fertig gebildet in den Gallensteinen angetroffen habe. Kommt es überhaupt darin vor, so kann es nur spurweise darin vorhanden sein. Wahrscheinlich verwandelt es sich in der alkalischen Galle durch Wasseraufnahme in Biliprasin. 85

Städeler purified and analyzed *Bilifuscin*. He washed out occluded fatty acids with ether and found the pigment was no longer soluble in $CHCl_3$, which allowed traces of $CHCl_3$-soluble bilirubin to be removed. After dissolution in alcohol and filtration, evaporation gave the pigment as an almost black lustrous brittle mass. Pulverizing afforded a dark brown powder with a somewhat olive color in it. It proved to be free of ash content, behaved like bilirubin upon heating, and gave a beautiful *Gmelin* reaction with HNO_3. From its combustion analysis (note the missing nitrogen analysis), *Städeler* determined the formula as $C_{32}H_{20}N_2O_8$, which suggested (to him) a close relationship to bilirubin, *i.e.* the two pigments differed by only two equivalents of water (*135*):[42]

[42] N.B. To *Städeler*, the formula for water in 1864 was HO, not H_2O.

So dargestellt bildet das Bilifuscin eine fast schwarze glänzende spröde Masse, die beim Zerreiben ein dunkelbraunes, etwas in's Olivenfarbene ziehendes Pulver giebt. Es ist frei von Aschenbestandtheilen, verhält sich beim Erhitzen eben so wie das Bilirubin und giebt mit Salpetersäure eine eben so schöne Pigmentreaction.

0,2655 Grm. der bei 120° getrockneten Substanz gaben bei der Verbrennung 0,614 Kohlensäure und 0,1575 Wasser; überesinstimmend mit der Formel $C_{32}H_{20}N_2O_8$:

			berechnet		gefunden
32	Aeq.	Kohlenstoff	192	63,16	63,07
20	„	Wasserstoff	20	6,58	6,59
2	„	Stickstoff	28	9,21	–
8	„	Sauerstoff	64	21,05	–
			304	100,00.	

Der Analyse zufolge steht das Bilifuscin in sehr einfacher Beziehung zum Bilirubin; es unterscheidet sich davon in der Zusammensetzung nur durch die Elemente von 2 Aeq. Wasser, welche es mehr enthält:

$\underbrace{C_{32}H_{18}N_2O_6}_{\text{Bilirubin}}$ $\underbrace{C_{32}H_{20}N_2O_6}_{\text{Bilifuscin.}}$

86

Indicating that *Bilifuscin* was, of all the pigments in gallstones, present in the smallest quantity, which perhaps placed a constraint on obtaining a %N in the combustion analysis, *Städeler* found just enough to learn a few of its properties: *Bilifuscin* was insoluble in H_2O, ether, and $CHCl_3$ (or only soluble in trace amounts); it was soluble in alcohol (giving a dark-brown color) which in high dilution had the color of strongly pigmented icteric urine, did not change color upon addition of HCl but became strongly reddish-brown upon addition of alkali. It dissolved easily in aqueous NH_3 or NaOH, producing a dark-brown solution from which brown flakes precipitated upon addition of HCl. Mixing an aq. NH_3 solution with $CaCl_2$ precipitated dark-brown flakes, much less voluminously than with bilirubin. Aerating an aq. NaOH solution of *Bilifuscin* caused decomposition, with color changes indicating the formation of *Biliprasin* and then probably *Bilihumin*.

The *Biliprasin* isolated from gallstones was also purified and analyzed by *Städeler*. It was pulverized, washed with ether and with $CHCl_3$, and then dissolved in cold alcohol and filtered. After evaporation of the dark green solution the "pure" *Biliprasin* was obtained as a lustrous, nearly black, brittle crust that looked quite similar to *Gallenbraun*. When pulverized it had a greenish-brown color. It yielded 0.6% ash upon combustion, which gave a strongly alkaline reaction and no effervescence with acids. The combustion analysis, correcting for ash, gave the formula $C_{32}H_{22}N_2O_{12}$, and the deviation from the calculated %N was not viewed as unusual, given the small amount of material available (*135*):

0,301 Grm. des bei 100° getrockneten Farbstoffes gaben bei der Verbrennung 0,627 Kohlensäure und 0,1765 Wasser.

Der Stickstoff wurde auf gleiche Weise bestimmt, wie beim Bilirubin. 0,096 Gm, gaben 0,073 Chlorsilber.

Diese Verhältnisse führen zu Formel $C_{32}H_{22}N_2O_{12}$:

			berechnet		gefunden
32	Aeq.	Kohlenstoff	192	56,81	56,81
22	„	Wasserstoff	22	6,51	6,52
2	„	Stickstoff	28	8,28	7,42
12	„	Sauerstoff	96	28,40	29,25
			338	100,00	100,00

Die Abweichung im Stickstoffgehalt ist nicht auffallend, wenn man berücksichtigt, dass zu dem Versuch nur eine sehr kleine Menge des Farbstoffes zu Gebote stand. 87

Städeler summarized the properties of *Biliprasin*: insoluble in H_2O, ether, and $CHCl_3$; soluble in alcohol to give a pure green coloration different from that of biliverdin, which had more of a blue-green color. These two pigments could thus be differentiated on the basis of the color of their solutions in alcohol (presumably at the same concentration) and the color change that ensued upon addition of ammonia: The *Biliprasin* solution turned brown; whereas, that of biliverdin did not. *Biliprasin* exposed to air absorbed some ammonia and dissolved in alcohol with a brown color, which could be confused with a *Bilifuscin* solution. For differentiation, the latter did not change color upon addition of HCl; whereas, the former became a beautiful green. As with bilirubin, biliverdin, and *Bilifuscin*, a positive *Gmelin* reaction was seen after mixing an alcohol solution of *Biliprasin* with HNO_3, except the blue color was recessive or indistinct. Although *Biliprasin* was easily soluble in alcohol, it was much less soluble in aq. Na_2CO_3. Highly dilute solutions had the same color as intensely brown pigmented icteric urine. If the solution were mixed with acid, the green color reappeared by removal of the alkali. Since brown, icteric urine showed the same color change upon acidification, one might conclude that *Biliprasin* was present in predominant amounts. Introducing air to a solution of *Biliprasin* in aq. NaOH caused it to go over gradually to *Bilihumin*.

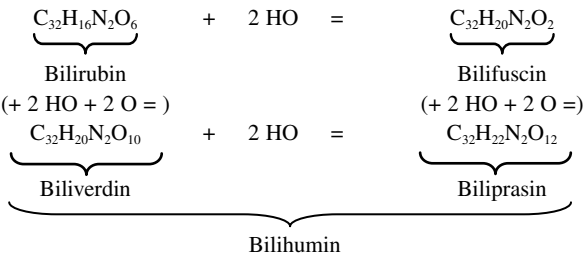
Bilihumin was found in a considerable quantity in gallstones and was not extracted into $CHCl_3$, ether, alcohol, H_2O, or dilute acid. It was freed completely of the various pigments already discussed by extraction a few times with aq. NH_3 to leave behind a black-brown pulverizable substance, which of course was not sufficiently pure for analysis. Purification was undertaken by repeated digestion in conc. ammonia at 50°–60° to extract a dark brown color and leave behind a dark brown solid that when dried and pulverized was black. The ammonia extracts were tediously processed by the usual methods: precipitation, washing, *etc*. to free the *Bilihumin* of inorganics. Yet despite multiple processing steps, *Städeler* did not consider the *Bilihumin* sufficiently pure for combustion analysis (*135*):

Eine Elementaranalyse habe ich nicht gemacht, da ich nicht die Ueberzeugung gewinnen konnte, dass der Körper rein sei, und da zu weiteren Reinigungsversuchen das vorhandene Material nicht ausreichend war. Ich bemerke nur, dass das gereinigte Bilihumin in Ammoniak nicht vollständig oder doch sehr langsam lösich ist, dass es sich dagegen in verdünnter Natronlauge beim Erwärmen ziemlich leicht löst, und dass die tiefbraune

Lösung, wenn sie mit Weingeist und dann mit NO_4[43] haltiger Salpetersäure vermischt wird, einen ganz hübschen Farbenwechsel zeigt. Namentlich ist das Roth sehr rein und intensiv, während die vorher auftretenden Farben in der tiefbraunen Lösung nicht deutlich zu erkennen sind. 88

The *Bilihumin* so obtained was found to be soluble in dilute aq. NaOH (but not in ammonia) to give a dark brown solution, which, upon addition of alcohol and HNO_3 containing NO_4 [= NO_2], gave a nice but different color change. Though the red color was very pure and intense, the preceding colors were not distinctly recognized in the dark brown solution.

Städeler said that *Bilihumin* captured his interest chiefly because it occurred as the final decomposition product of all the rest of the bile pigments when, in aq. NaOH solution, they were exposed to air. He then proposed a simple relationship between *Bilihumin* and the others (*135*):



Ohne Zweifel steht die Formel des Bilihumins in einem ähnlichen Verhältniss zu der des Biliprasins, wie die Formeln der analysirten Körper unter einander. Für sehr wahrscheinlich halte ich es auch, dass die im lebenden Organismus vorkommenden dunkelen unlöslichen Pigmentsubstanzen, das s.g. *Melanin*, sich dem Bilihumin anschliessen und vielleicht gleichen Ursprungs sind. 89

And he suggested the likelihood that dark, insoluble pigments, the so-called *Melanin*, reminded one of *Bilihumin* and perhaps originated from the same source.

Although the focus of *Städeler*'s comprehensive work was the pigments of gallstones, he also looked into human bile in order to reinvestigate similarities between his bilirubin and *Valentiner*'s hematoidin, and to address further *Frerichs'*, *Neukomm*'s, and his own notion of bile acids as a source of bile pigment. *Städeler* knew that there was little doubt (and no further proof was needed) that the bilirubin of human gallstones came from inspissation of the same pigment in human bile. It was the apparent differences in crystal form between bilirubin and hematoidin that interested him, and he suspected that the crystal form was promoted by impurities carried along in the pigment isolation. He reasoned that if bilirubin and hematoidin were in fact identical, a more careful extraction and purification would confirm it (*135*):

[43] N.B. $NO_4 = NO_2$ when the atomic weight of oxygen is changed to 16 from *Gmelin*'s system of "equivalents", where O = 8.

Die menschliche Galle.

Es bedarf keiner chemischen Beweisführung, um die Annahme zu rechtfertigen, dass in der menschlichen Galle dieselben Farbstoffe vorkommen, wie in den Concrementen, welche sich darin bilden. Die Versuche, welche ich mit menschlicher Galle angestellt habe, hatten daher einen anderen Zweck. Wie bereits erwähnt, ist die krystallinische Form des Bilirubins um so mangelhafter, je reiner die Lösungen sind, aus welchen es anschiesst, während unreine Chloroformlösungen ganz gewöhnlich krystallinisches Bilirubin liefern. Die krystallinische Ausscheidung scheint bedingt zu sein oder doch sehr befördet zu werden durch die Gegenwart gewisser fremder Stoffe, ebenso wie zur krystallinischen Ausscheidung des Teichmann'schen Hämins aus essigsauerer Lösung die Gegenwart irgend welcher Chlormetalle erforderlich ist. Ich wählte daher die Galle, um das Bilirubin in messbarer Form darzustellen. War der darin vorkommende rothe Farbstoff wirklich identisch mit dem Hämatoïdin, wie Valentiner annimmt, so musste er sich bei richtig gewählter Behandlung auch in der so regelmässig auftretenden Hämatoïdinform gewinnen lassen. 90

Städeler had learned from his own experiments that in addition to its solubility in $CHCl_3$, bilirubin was sufficiently soluble in CS_2 and in benzene to be extracted from gallstones. Thus, he extracted human bile with these three solvents. From repeated experiments using $CHCl_3$, he obtained crystals of bilirubin that usually did not match up exactly with the crystal form of hematoidin, although in one experiment they came rather close (*135*):

Schüttelt man Galle mit Chloroform, so beobachtet man, wie schon Valentiner gefunden hat, beim langsamen Verdunsten der Lösung die Bildung von orangefarbigen elliptischen Blättchen oder sehr kleiner, fast rechtwinkeliger Tafeln, deren Winkelverhältnisse sehr wesentlich verschieden sind von denen des Hämatoïdins. Bei wiederholten Versuchen war das Resultat immer nahezu dasselbe; immer wurden jene rhomboïdischen Gestalten mit geringem Unterschiede der Seiten und Winkel wahrgenommen, bei denen die Diagonalen des Rhomboïdes durch abweichende Färbung markirt waren. Nur ausnahmsweise wurde mitunter einmal eine vereinzelte Form beobachtet, die sich der gewöhnlichen Hämatoïdinform näherte. 91

Using CS_2 and commercial benzene, which he purified by distillation, taking no fraction with a boiling point greater than 100°C and making sure as best he could that it contained no sulfur, he extracted bile. Actually the bile from two humans was dried, pulverized, and partitioned three ways into three flasks. One part was extracted or digested using $CHCl_3$, one by CS_2, and the third by the purified benzene, and in all of these washings a yellow coloration was produced. To each was added 20 drops of 25% aq. HCl, with continuous shaking, and after 12 hours each was filtered through filter paper moistened with the corresponding solvent. (It is not clear whether air was excluded in this procedure).

The $CHCl_3$ solution was colored an intense green and left a resinous violet residue upon passive evaporation. The residue was washed successively with ether (to remove cholesterol and fats) and alcohol (to remove a green pigment and other possible substances). The bilirubin so obtained consisted of orange-colored crystalline granules and flakes mixed with rhomboids described earlier (*135*):

Die *Chloroformlösung* hatte eine intensiv grüne Farbe und hinterliess beim freiwilligen Verdunsten einen mehr violetten klebenden Rückstand. Bei der Behandlung mit Aether wurden Cholesterin und Fett ausgezogen, Weingeist nahm neben anderen Substanzen den grünen Farbstoff auf, der nach seinem Verhalten gegen Alklien Biliverdin . . . zu sein schien, und als Rückstand wurde Bilirubin erhalten, aber nicht in guten Krystallen, sondern in orangefarbigen krystallinischen Körnern und Flocken, die mit den beschriebenen rhomboïdischen Formen gemengt waren. 92

The CS_2 extract had a pure golden yellow color, and after passive evaporation left behind a reddish crystalline mass. From the last, cholesterol, fats, and some bile acids were removed in the usual way to leave behind bilirubin as dark red microscopic crystals, which *Städeler* described in considerable detail. He found them different from the crystals obtained following the $CHCl_3$ extraction and while similar to those of hematoidin, he was unable to confirm an exact match due to the small size of his hematoidin crystals (*135*):

> Die *Schwefelkohlenstofflösung* hatte eine rein goldgelbe Farbe. Beim freiwilligen Verdunsten hinterliess sie eine röthliche krystallinische Masse, aus der Aether und Weingeist Cholesterin, Fett und vielleicht auch etwas Gallensäure aufnahmen, während das Bilirubin in tiefrothen mikroscopischen Krystallen zurückblieb. Die Krystalle erschienen als klinorhombische Prismen mit der Basisfläche, woran der vordere Winkel sehr scharf und die Prismenflächen convex gebogen waren, so dass die Ansicht auf die Basisfläche Ellipsen zeigte. Auf den convexen Flächen aufliegende Krystalle zeigten rhomboïdische Gestalten mit bedeutend grösserem Unterschiede der Seiten und Winkel, als bei den aus Chloroform angeschossenen Krystallen. Häufig findet man die prismatischen Krystalle in der Mitte eingeschnürt, was auf Zwillingsbildung hinzudeuten scheint. Die Diagonalen waren auf gleiche Weise markirt wie bei den aus Chloroform angeschossenen Krystallen. … – Die Winkelverhältnisse dieser Krystalle zeigten Aehnlichkeit mit denen des Hämatoïdins; genaue Messungen und Vergleichungen waren aber wegen der Convexität der Flächen und wegen der Kleinheit der mir zu Gebote stehenden Hämatoïdinkrystalle leider nicht möglich. 93

The benzene extract had the same color as the CS_2 extract and yielded a quite similar residue of crystals upon evaporation in a mildly heated water bath, but these bilirubin crystals were larger and more irregular. Yet even if the crystals from benzene and from CS_2 were similar to hematoidin, *Städeler* concluded that was not a sufficient basis to conclude that bilirubin and hematoidin are identical. He indicated that a sufficient basis had to come from their combustion analyses; which showed large differences – differences that he concluded could not possibly be due to a small impurity or an unavoidable analytical error (*135*):

> Zunächst sind beim Hämatoïdin noch niemals convexe Flächen beobachtet worden, während dieselben beim Bilirubin so hervortretend sind, dass man dasselbe bei flüchtiger Betrachtung leicht für Harnsäure halten könnte. Das Hauptgewicht muss aber auf das Resultat der Analyse gelegt werden, und da ergiebt sich, wie die folgende Zusammenstellung zeigt, eine so grosse Abweichung in der Zusammensetzung, dass man die Differenz unmöglich auf Rechnung geringer Verunreinigungen* oder der unvermeidlichen Analysenfehler setzen kann.

	Bilirubin		Hämatoïdin	
Kohlenstoff	67,15	67,11	65,85	65,05
Wasserstoff	6,27	6,12	6,47	6,37
Stickstoff	9,59		10,51	
Sauerstoff	16,99		17,17	
	100,00		100,00	

*Bei einem nicht genügend gereinigten Bilirubin fand ich folgende procentische Zusammensetzung: 66,52 Kohlenstoff, 6 Wasserstoff, 8,7 Stickstoff und 18,78 Sauerstoff. 94

A few years earlier, *Städeler* had called attention (cited in *120*) to the fact that the formula from *Robin*'s combustion data (*115*, *116*) did not agree with the formula $C_{14}H_9NO_3$ but that the formula $C_{30}H_{18}N_2O_6$ did, although it was out of correspondence by 0.1% and 0.2% smaller in hydrogen. *Städeler* concluded that a close relationship existed between bilirubin and hematoidin based on the great similarity of their formulas: If hematoidin contained two fewer hydrogens, its formula would thus be $C_{30}H_{16}N_2O_6$; so, it and bilirubin ($C_{32}H_{18}N_2O_6$) would belong to a homologous series, which would clarify their manifold similarities in characteristics. *Städeler* believed that a decision could be reached only from new combustion analyses (*135*):

> Robin . . . hat aus jenen Analysen die Formel $C_{14}H_9NO_3$ für das Hämatoïdin berechnet, doch habe ich schon vor Jahren darauf aufmerksam gemacht . . . , dass diese Formel nicht mit Robin's Analysen übereinstimmt, und dass man bei richtiger Berechnung zu der Formel $C_{30}H_{18}N_2O_6$ gelangt; nur der Wasserstoff ist in diesem Falle um 1/10 und 2/10 pC. geringer gefunden, als der Formel entspricht. – Dass Bilirubin und Hämatoïdin nahe verwandte Körper sind, ergiebt sich schon aus der grossen Aehnlichkeit der Formeln. Enthielte das Hämatoïdin 2 Aeq. Wasserstoff weniger, hätte es also die Formel $C_{30}H_{16}N_2O_6$, so würde es mit dem Bilirubin, $C_{32}H_{18}N_2O_6$, in eine homologe Reihe gehören, und damit wären die mehrfachen Aehnlichkeiten in den Eigenschaften genügend erklärt. Doch darüber kann nur durch neue Analysen entschieden werden. 95

Städeler's comprehensive publication on bilirubin from gallstones and bile would not have been complete without his concluding comments directed toward other pigments that gave a positive *Gmelin* reaction. Such included the green pigments that he isolated from gallstones as indicated above: biliverdin and *Biliprasin*, another green pigment isolated by *Scherer* from icteric urine (*92*, *93*), which *Städeler* thought was a decomposition product formed in the isolation procedure (*135*):

> Wahrscheinlich war dieser Farbstoff ebenfalls nur ein Zersetzungsproduct, entstanden durch Einwirkung der Salzsäure auf den ursprünglichen Farbstoff; jedenfalls war er nicht rein, wie aus dem hohen Kohlenstoff- und Wasserstoffgehalt neben dem geringen Stickstoffgehalt hervorgeht. 96

All these and a third green compound isolated several years earlier by *Städeler* (when the $CHCl_3$ extraction method was not known) from a brown-colored ox gallstone the size of a walnut that had been given to him by his friend Prof. *Merklein* in Schaffhausen, Switzerland. This green material gave the formula $C_{32}H_{18.5}N_{2.5}O_{10}$ from combustion analysis – an odd analysis that *Städeler* attributed to insufficient care that a pure sample was used. He believed that gallstones from animals appeared to be richer in nitrogen (10.5% N) than those from humans.

Städeler could not end the discussion of the pigments of gallstones and bile without a commentary on "synthetic" bile pigments that also show a beautiful color change, the *Gmelin* reaction. He had obtained a brownish-red pigment by warming a bile salt in conc. H_2SO_4, a chromogen that precipitated in resin-like flakes upon addition of H_2O. If the H_2SO_4 solution were warmed briefly in the absence of air, the precipitated flakes were colorless or greenish, but after standing 24 hours in conc. H_2SO_4 the solution showed a beautiful dichroism that was orange-colored or brownish with a striking pure green transmitted light. Addition of H_2O precipitated green-blue flakes. Further processing, isolation, and purification produced a pigment that imparted a bile-green color in alcohol, became yellow or orange upon basification, and returned to green upon addition of HCl. With "NO_4"-containing

HNO_3 (presumably it was NO_2; NO_4 assumes the atomic weight for oxygen is 8), the pigment gave, even at great dilution, a vivid color change: at first green, then green-blue or greenish-brown, next red, and finally dirty yellow. These pigment color reactions appeared (to *Städeler*) to signify a relationship between the synthetic and natural pigment. With this perspective, *Städeler* thought it not inappropriate to think that the bile pigments found in the urine of dogs after intravenous injection of bile acids came from their transformation in the blood stream. This "completely proven and irrefutably established fact" was not brought to the fore as such, however, because bile pigments were not detected in bile in some experiments after intravenous administration of bile acids. Yet there was also an unresolved question as to how a nitrogen-free bile acid might be converted to a nitrogen-containing bile pigment (*135*):

> Da durch diese Pigmentreaction ein Zusammenhang der künstlichen Pigmente mit den natürlichen Gallenpigmenten angedeutet schien, und da wir, wie schon oben (S. 324 f.) angegeben wurde, ausserdem noch beobachteten, dass nach der Injection von gallensauren Salzen in eine Vene fast regelmässig Gallenpigment im Harn auftritt, so war es gewiss nicht übereilt, wenn wir schlossen, dass die Gallensäuren auch in der Blutbahn eine Umwandlung in Pigment erleiden könnten. Als völlig erwiesene und unumstösslich feststehende Thatsache ist diese Umwandlung übrigens niemals hingestellt worden, da uns einige, wenn auch nur wenige Fälle vorkamen, wo nach Galleninjection kein Pigment im Urin nachgewiesen werden konnte. Es ist mir jetzt gelungen, auch die stickstofffreie Cholsäure auf gleiche Weise wie die Glycocholsäure und Taurocholsäure in Farbstoffe zu verwandeln, und da sich ungezwungen nicht annehmen lässt, dass die stickstoffhaltigen Gallenpigmente ihr Entstehen einem stickstofffreien Körper verdanken, so kann von einer Umwandlung der Gallensäuren in die wirklichen Gallenfarbstoffe nicht wohl ferner mehr die Rede sein. 97

And there was an equally fundamental question related to whether the bile pigments found in urine under the circumstances described come about by transformation of the intravenously injected bile acids or whether the red cells of blood were lysed by the bile acid and their extruded pigment was the source of the urinary bile pigments. Arguing against the latter is that injection of H_2O did not lead to bile pigments and that, in the case of a rabbit, injection of water produced urine that was rich in blood pigment but contained no bile pigment (*135*):

> Es bleibt nun noch immer die Frage unerledigt, welche Rolle die in das Blut getretene Galle bei der Erzeugung der Gallenpigmente spielt; denn die Annahme, dass die Gallensäure *nur* die Blutkörperchen auflöst, und dass das gelöste Blutroth dann in Gallenfarbstoff übergehe, scheint mir doch nicht gerechtfertigt zu sein. Einmal müsste dann nach Galleninjectionen regelmässig Gallenpigment im Urin auftreten was bekanntlich nicht der Fall ist, und ausserdem müssten Wasserinjectionen dieselbe Wirkung hervorbringen wie die Injection von Gallensäuren. Auch dieses ist nicht der Fall. Röhrig . . . spritzte einem Kaninchen, dessen Blutgehalt sich zu 130 Grm. berechnete, 100 CC. Wasser in die Vena jugularis und beobachtete, dass der darauf gelassene Harn reich an Blutpigment war, aber keinen Gallenfarbstoff enthielt. 98

A new idea apparently struck *Städeler* when he realized that during icterus the heartbeat was known to be reduced, usually by 20–30 contractions. His colleague *Frerichs* mentioned this and cited two cases where the heartbeat dropped 28 and 21 beats. He ascribed the perturbations to the presence of bile acids and suggested that small amounts of sodium salts of glycocholic, taurocholic, and cholic acids act likewise, proportionately depressing the pulse. The presence of larger amounts of bile acid salts led to sudden death by paralysis of the heart.

Yet on the basis of all the various observations made toward understanding the induction of bile pigments in urine, which *Städeler* knew from his and *Neukomm*'s studies that the bile pigments were not always found in urine post intravenous injection, doubts persisted. Other factors may have been the cause: differences in age, size, and constitution of the dogs used were uncontrolled potential variables, as was the heartbeat (*135*):

> Nach diesen Beobachtungen halte ich es für wahrscheinlich, dass wir in diesen enormen Kreislaufstörungen, mit denen natürlich auch grosse Störungen in der chemischen Stoffmetamorphose verbunden sein müssen, hauptsächlich den Grund der Pigmentbildung nach Einführung von Gallensäuren in das Blut zu suchen haben. Es würde sich damit auch erklären, dass die Pigmentbildung nicht constant eintritt, denn Thiere von verschiedenem Alter und Grösse, von schwacher und kräftiger Constitution, können nicht auf gleiche Weise von derselben Menge Gallensäure afficirt werden. – Demnach wäre also die Pigmentbildung nach Galleninjection nur eine secundäre Wirkung der in's Blut gebrachten Gallensäure, und ist dieses der Fall, so steht zu erwarten, dass andere Substanzen, welche ähnlich Störungen der Herzthätigkeit hervorbringen, ebenfalls zur Bildung von Gallenpigment Veranlassung geben müssen. Eine solche Substanz besitzen wir in der Digitalis, mit der ich einige Versuche angestellt habe. 99

For the last, *Städeler* reasoned that if a bile acid-perturbed heartbeat were the root cause of the presence of urinary bile pigments, he might conduct control experiments using digitalis as a heartbeat perturber. (Of course, as with bile acids, the chemical structure of the steroid digitalis was also not known.) So he brought two dogs up to a modicum of good health, and after their urine proved to be free of bile pigment, he then infused the animals with 2 g of herbal digitalis – which induced vomiting and diarrhea. Some 48 hours later, the urine of one dog showed a distinct and intense pigment reaction with HNO_3. Using the $Pb(OAc)_2$ precipitation method to sequester the pigment, a positive *Gmelin* reaction was confirmed for eight days following the initial dose. The second dog gave no detectable bile pigment in urine following the same procedure as in the first dog. The poor dogs expired eight days following the initial dosing.

Städeler admitted that these contradictory results did little to settle the issue, which was apparently still unresolved, at least from his perspective, and he was resigned to the belief that a large series of experiments would be required. Then he essentially bowed out of bile pigment research by indicating that other work prevented his giving the question the attention it deserved (*135*):

> Diese beiden Versuche widersprechen einander. Die angeregte Frage ist also noch nicht erledigt; sie lässt sich aber nur durch eine grössere Versuchsreihe beantworten, und ich bedauere, dass andere Arbeiten mich verhindern, diesem Gegenstande ferner die Aufmerksamkeit zu widmen, die er zu verdienen scheint. 100

To summarize *Städeler*'s achievements briefly, he introduced new names for bile pigments isolated from gallstones: (i) *Bilirubin*, for the reddish pigment of gallstones and bile (*Gallenroth*), which soon thereafter replaced the older names *Cholepyrrhin* [*Berzelius*' yellow pigment from bile (*73–76*)], and *Biliphäin* [*Simon*'s name for *Cholepyrrhin* (*89–91*)], and the contemporary name *cholophain* or *Cholophäin* [*Thudichum*'s name for *Cholepyrrhin* or *Biliphäin* (*103*)]; and (ii) *Bilifuscin* and *Biliprasin* (brownish pigments), *Bilihumin* (brownish-green). The only original name that has persisted, *Biliverdin*, is that given by *Berzelius* to the green pigment of bile (*Gallengrün*), and which *Städeler* also isolated from gallstones.

In 1864, *Städeler* undoubtedly had prepared the purest bilirubin up to that time, taking care in the $CHCl_3$ extractions that the solvent was freed of HCl (from its decomposition to $COCl_2$ and HCl by light). He also learned by so doing that the pigments of gallstones clung to certain metals, as salts, mainly to calcium. This fact may have been suspected by the investigators immediately preceding him such as *Brücke* (*109*), *Heintz* (*97*), and *Hein* (*105*). They had to exert considerable effort to prepare pigment samples from bile and gallstones that were ash-free by combustion – a difficulty that plagued elemental combustion analyses prior to *Städeler* – causing him difficulty in his *Biliprasin* analysis and thwarting an analysis of *Bilihumin*.[44]

The combustion analysis data (see Table 2.9.1) obtained by *Städeler* differed from the data of his earlier (*120*) analyses of bilirubin, and as indicated earlier, he had insufficient biliverdin for analysis. As did his predecessors, from the %C, H, N data *Städeler* calculated formulas for the pigments – and he made many attempts to provide correlations between the pigments based on these formulas. Although well-intentioned in this, *Städeler* and others preceding him struggled with sample purity, which is always a consideration, and were dependent on, unknowingly (and hamstrung by), the prevailing assignments of the atomic weights of C, H, N, and O.

Table 2.9.1 *Städeler*'s elemental combustion analysis data of bilirubin, *bilifuscin*, and *biliprasin* compared with hematoidin. (The formulas are based upon the *Gmelin* system of atomic equivalents, C = 6, H = 1, N = 14, and O = 8)

	Bilirubin					Hematoidin			
	Experimental			Calculated for		Experimental[c]		Calculated for	
%	A[a]	B[a]	C[b]	$C_{32}H_{18}N_2O_6$[a]	$C_{18}H_9NO_4$[b]	D	E	$C_{30}H_{18}N_2O_6$[d]	$C_{14}H_9NO_3$[e]
C	67.15	67.11	66.52	67.13	66.26	65.85	65.05	65.69	64.12
H	6.27	6.12	6.00	6.29	8.52	6.47	6.37	6.57	6.87
N	9.59	–	8.70	9.79	8.59	10.50	10.50	10.22	10.69
O	16.99	–	18.78	16.79	19.63	17.18	18.08	17.52	18.32

	Bilifuscin[a]		*Biliprasin*[a]		*Biliverdin*[a]	
%	Experimental	Calculated for $C_{32}H_{20}N_2O_8$	Experimental	Calculated for $C_{32}H_{22}N_2O_{12}$	Experimental	Calculated for $C_{32}H_{20}N_2O_{10}$
C	63.07	63.16	56.81	56.81	60.00	60.04
H	6.59	6.58	6.52	6.51	6.25	5.84
N	–	9.21	7.42	8.28	8.75	8.53
O	–	21.05	29.25	28.40	25.00	25.59

[a] *Städeler's* data from reference (*135*)

[b] *Heintz's* data from reference (*97*)

[c] Hematoidin experimental data from *Robin* (*115*, *116*)

[d] *Städeler's* formula (*135*) from *Robin's* experimental data

[e] *Robin's* formula from his experimental data (*115*, *116*)

[44] N.B. Combustion analyses may at times be more important in revealing the presence of impurities than in characterizing the intended compound.

The issues surrounding atomic weights were addressed on September 5, 1860, at a major European and first *international* scientific congress which opened in Karlsruhe, capital city of the Grand Duchy of Baden. (Karlsruhe entered the German empire in 1871 and is now part of the German Federal Republic State of Baden-Württenberg.) The congress was organized by *Kekulé*, *Wurtz*, *Weltzien*, *Baeyer*, *Roscoe*, and *Williamson* to discuss the major issues in science (*147-149*). Among the topics were the highly disputed atomic weights, especially those of C and O, which are of great importance to organic chemistry and the then undeveloped Periodic Table of Elements. According to *Kauffman* and *Adloff*, writing on the history of the Karlsruhe Congress, as an alternative to *Dalton*'s "incorrect and inadequate" atomic weights (relative atomic masses), the year 1814 brought forth (from *W.H. Wollaston*) (*149*):

> a new, more pragmatic term, "atomic equivalent". . . . In dealing with proportional relationships between chemical compounds many chemists such as Leopold Gmelin used equivalent weights (He called them "*Mischungsgewichte*") . . . rather than atomic weights. The resulting debate between the so-called "atomists" and "equivalentists" raged for another half century.
>
> Until 1849, when English chemist Edward Frankland (1825-1899) recognized the concept of valence, . . . it was impossible to know whether the assigned atomic weights were correct or should be multiples of the values. Thus, for example, some chemists used atomic weights of 6 and 8 for carbon and oxygen, respectively, while others preferred atomic weights of 12 and 16. Therefore different formulas were often assigned to the same substance. . . . As an extreme example of the problem of inconsistent formulas we may cite the 19 formulas for acetic acid in August Kekulé's organic chemistry textbook of 1861 [*28*]…

In the third session of the Congress the conflicting theories and concepts were addressed in a lecture by *Cannizzaro*, who reminded the attendees of *Avogadro*'s hypothesis (equal volumes of gases at the same temperature and pressure have the same number of molecules) and on that basis, as well as the Law of *Dulong* and *Petit* (relating the specific heat of a solid to its atomic weight), reassigned the atomic weight of C from 6 to 12, O from 8 to 16, S from 16 to 32, *etc*. while convincing the majority of the attendees (*149*):

> The Karlsruhe Congress dramatized the importance in the minds of the younger attendees of Avogadro's hypothesis, . . . which had been largely overlooked for half a century, thus making possible the impressive strides in chemistry that took place during the next four decades of the nineteenth century. Removing the uncertainty about atomic weights established the certainty of molecular weights and made it possible to distinguish between empirical and molecular formulas and to formulate correctly hydrocarbons, alcohols, organic acids, aromatic compounds, and almost all the simpler organic molecules, leading to the tremendous progress in organic chemistry. . . .

Though the new (correct) set of atomic weights was well-accepted in Germany, it lagged in some other countries. Yet its influence was considerable (*149*):

> The congress established a paradigm shift for the understanding of chemistry and led to the periodic tables of Mendeleev . . . and Lothar Meyer. . . .
>
> In addition to its impact on the development of chemical theory and practice discussed above, the Karlsruhe Conference was the prototype for future international chemical meetings.

Although the change was adopted only slowly, *Städeler*'s formulas were based on the relative atomic weights H = 1, C = 6, N = 14, and O = 8. Which explains his use of the formulas HO (or OH) for H_2O and NO_4 for NO_2 that should look odd to us today but illustrate how much the structure of the chemical sciences depends on exact fundamental constants.

Finally, *Städeler* continued to address the apparent (to him) transformation of bile acids into "synthetic" bile pigments that give an apparent positive *Gmelin* color reaction. He demonstrated this by transformation in two ways: in conc. H_2SO_4 from which he isolated the pigment, and detection in urine following intravenous injection of a bile acid. However, the latter experiment sometimes produced an apparent bile pigment and other times it did not, which created uncertainty. *Städeler* was clearly a careful scientist in analyzing the experiment and hesitated to commit firmly to the thesis, while also questioning how the transformation of a bile acid that contained no nitrogen might be transformed to a bile pigment that does. The 1864 publication was apparently his last on the subject of bile pigments, and he was to die some seven years later.

2.9.2 *Johann Ludwig Wilhelm (aka John Lewis William) Thudichum and Bilirubin*

Thudichum[45] cast a broad shadow across the entire gallstone literature in the last half of the 19th century, including the chemistry of gallstones. Unlike *Städeler*, *Thudichum* lived a long life. In 1863, he wrote a long treatise on gallstones (*103*), citing the history of the early chemical analyses, the older analytical proceedings of

[45] *Johann Ludwig Wilhelm* (*aka John Lewis William*) *Thudichum* was born eight years after *Städeler*, on August 27, 1829 in Büdingen, in Hessen, Germany, and died on September 7, 1901 in Kensington, in his adopted England. Though he is most noted for his studies on the chemical constitution of the brain (identifying sphingomyelin, sulfatides, cerebrosides, *etc*. therein) in the late 1800s, his fame came mainly posthumously. His greatest work, A *Treatise on the Chemical Constitution of the Brain*, stirred controversy and provoked criticism for his rejection of the then firm belief that the brain is composed of a single giant molecule (Protagon) and his insistence that it consisted of elaborate chemical structures (in the scientific press he was called by some a liar and falsifier). At age 18, he began medical studies in 1847 at the University of Giessen, working after hours in *Justus Liebig*'s lab, where he developed his interest in physiological chemistry. He studied in Heidelberg, volunteered as a surgeon in 1850 during the Prussian-Danish War, then obtained the Dr. med. degree in 1851 at Giessen, where he began his medical practice. Drawn to chemistry from his studies under *Liebig*, and at odds politically over the war, he emigrated to London in 1853, where he obtained the diploma M.R.C.S. Eng. in 1854 and where he practiced medicine as an otologist and rhinologist first at St. Pancras Dispensary and elsewhere. After accepting several subsequent appointments, in 1860 he became M.R.C.P. and in 1865 was appointed Lecturer at St. Thomas's Hospital and director of its newly founded chemical and pathological laboratory. While continuing his medical practice, from 1871 he conducted experimental physiological chemistry in his home laboratory. In addition to his aforementioned work on the brain, he wrote authoritative treatises on urine and on gallstones.

Berzelius and *Heintz*, and a method of his own for analyzing human gallstones. For reasons not entirely clear, he coined new names for pigments: The "colouring matter of bile and all its varieties" he called "cholochrome"; the brown coloring matter he retained the name "cholophæine" (synonymous with *Cholepyrrhin*, *Biliphäin*, and *Bilifulvin*); for the green he adopted the name *cholochloine* (synonymous with *Biliverdin* and *Cholechlorin*). Just why a new set of names was required is unknown (except possibly to put his stamp on the bile pigment field?), but they fortunately began to melt away some five years later when he began to use the new term *Bilirubin* in a major publication on bile pigments (*152*). Previously he wrote briefly on the composition of gallstones (*102*), with separations based on modifications of *Berzelius'* approach some two decades earlier (*71*, *72–76*) by treating pulverized gallstones with H_2SO_4, followed by precipitating with or without $Ba(OH)_2$, $(NH_4)_2S$, acidifying with HCl, basifying with NH_4OH and decanting as needed along the separation route, *etc*. to provide cholochrome and inorganic salts, *inter alia*.

In his comprehensive treatise on gallstones (*103*), *Thudichum* reviewed the early experiments of his predecessors who attempted to separate the components of bile, from work preceding that of *Haller* (*35*) in 1764 to the more recent studies of *Fourcroy* (*42*) and *Thenard* (*53–56*), *Berzelius* (*68–76*), and *Heintz* (*95–97*), who was concerned about the amount of ash remaining in his combustion analyses of bile pigments. *Thudichum*, too, was especially concerned with combustion analyses and composition; he repeated the analysis of cholophæine (= *Cholepyrrhin* = *Biliphäin*) of material isolated from gallstones according to *Heintz* only to obtain different results for the %C, and even larger differences in %C from material isolated from ox bile (*103*):

> . . . Since the first attempts of Berzelius, about 1812, to determine the properties of the colouring matter of bile, several analyses have been instituted with the particular object of ascertaining its chemical or elementary composition. Those of Scherer (1843), Hein (1847), Heintz (1854), and Städeler (1861), were the most methodical, although none of them have led to final results. The elementary analyses of Scherer and Hein were performed upon specimens of cholochrome which, to conclude from the process adopted for their preparation, must have contained impurities and inorganic matter. The analyses of Heintz, on the contrary, were executed upon materials apparently homogeneous, and certainly free from inorganic substances. But the analyses of cholophæine, the brown modification of cholochrome, lead to a formula which is very ill-supported by the formula of the only metamorphosis to which, at that period, cholophæine could be subjected. Four elementary analyses, agreeing with each other, led to the empirical formula $C_{31}H_{18}N_2O_9$ for cholophæine; but one analysis of cholochloine, the green colouring matter hitherto termed biliverdine, obtained from the brown by oxidation, led Heintz to the formula $C_{16}H_9NO_5$. The improbability of the suggestion that cholophaeine, in order to pass into cholochloine, should take up only half an equivalent of oxygen, Heintz met by assuming the formula of cholophæine to be $C_{32}H_{18}N_2O_9$, and by further assuming that this body took up one equivalent of oxygen, and then split up into two equivalents of cholochloine.
>
> I have repeated the analysis of cholophæine upon materials prepared in accordance with the precedent of Heintz. In some of them I have obtained figures which are very near to those of Heintz, the hydrogen in most cases keeping steadily near 6 per cent.; but the carbon varied between 60 and 62 per cent., or to the same extent to which the first analyses of Heintz differed from his check calculation. But when I came to analyse cholophæine obtained from ox bile directly (the former specimens having been prepared from gall-stones), I obtained totally different results, the carbon rising to 66, the hydrogen to 10 and 11 per cent.

The only combustion analyses data directly cited were those of *Städeler* for purified *Cholepyrrhin*, obtained from gallstones by $CHCl_3$ extractions, and for which the formula $C_{18}H_9NO_4$ was calculated (*103*):

> An elementary analysis by Städeler of cholepyrrhine, purified by repeated crystallization from boiling and washing with cold chloroform, yielded results from which the formula $C_{18}H_9NO_4$ was calculated.

				Calculated.		Found.
18	equivalents of	carbon	=	108	66·26	66·52
9	"	hydrogen	=	9	5·52	6·00
1	"	nitrogen	=	14	8·59	8·70
4	"	oxygen	=	32	19·63	18·78
				163	100·00	100·00

Confirming the absence of iron as a component of his cholochrome, *Thudichum* reached a rash but perhaps a logical conclusion then, but unjustified now, that there is, on the basis of the absence of iron in the bile pigment, no apparent connection between it and the pigment of blood (which was known to contain iron) (*103*):

> In none of the specimens analysed by me was there any trace of iron; I can, therefore, fully confirm the statement of Heintz, that iron is not an elementary ingredient of cholochrome. Hence it follows that cholochrome has no immediately apparent connection with the colouring matter of blood.

Thudichum modified *Berzelius*' method for the separation of bile pigments from bile, in which bile was left standing 1–2 days before a tedious work-up that led to what he believed to be "somewhat impure cholechloine" (= biliverdin), a "beautifully green substance." From human and ox gallstones, he applied *Heintz*'s method to separate olive-green tinted and brown cholophæine that, as with *Heintz*'s, was "perfectly free from ash on combustion" (*103*). He then subjected a narrow, 15-inch-long tube of cholochrome (from ox gallstones) to extraction into $CHCl_3$ over a 1-week-period to yield a reddish-brown solution, from which he obtained red crystals, which he supposed to be the "original form of cholochrome or *cholerythrine*" – and which one might now assume was cholophæine = *Cholepyrrhin* = *Biliphäin* = hematoidin = bilirubin (*103*):

> . . . the chloroform of the extract was distilled off, and the concentrated solution left to spontaneous evaporation. A granular substance was deposited, which yielded to boiling absolute alcohol a greenish-brown matter, and became a most beautiful red colour, resembling cinnabar or red oxide of mercury. When dry, it had the sweet, musk-like odour of a healthy cow. Viewed under the microscope, it appeared mostly amorphous; but when a concentrated solution in chloroform was allowed to evaporate slowly under a little glass cover, crystals were formed in great numbers, being needles and rhombic plates. The powder was insoluble in water, little soluble in boiling absolute alcohol, sparingly soluble in ether, easily soluble in chloroform, a little more soluble in boiling than in cold chloroform. It was soluble in dilute solutions of caustic and carbonated alkalies and in an alcoholic solution of caustic potassa. When treated with concentrated sulphuric acid, it dissolved with a yellow colour, and green flakes separated on the addition of water. Nitric acid imparted a deep-crimson colour to the powder, dissolving a part, which changed from red

> to blue, violet, and lastly crimson. This change of colour was particularly beautiful on a thin layer of colouring matter, produced by allowing a very dilute chloroform solution to evaporate in a china dish. Such a layer, like a stain of the same solution on the skin, was of a bright-yellow colour.
>
> This red substance is, evidently, the original form of biliary colouring matter, and a chemically pure body. I shall hereafter speak of it as the red or original form of cholochrome or *cholerythrine*.

Consistent with earlier observations that the reddish pigment was easily oxidized to the green, *Thudichum* found that cholophæine was easily oxidized to a green pigment that he called *cholochloine*, then later called it *Biliverdin*. In a different oxidation, one initiated by nitrous oxide gas (N_2O) followed by HNO_3, there was isolated a new crystalline, water-insoluble substance, and an "uncrystallizable acid, which gave a crystallized salt with ammonia." The intermediate at the first step by treatment with nitrous acid, called *cholochromic acid* by *Thudichum*, was isolated apparently in two crystal modifications, if not two chemically-different reddish compounds. One showed the crystal form and color attributed to hematoidin, but neither type of crystal could be isolated from the surrounding syrup. Various manipulations of cholochromic acid were engaged: nearly insoluble in H_2O; soluble in spirit of wine, to give a port wine colored strongly acidic solution that precipitated a red solid with aq. $Pb(OAc)_2$; a pink solid with $AgNO_3$, turned deep red upon addition of ammonia. *Thudichum* concluded that cholochromic acid is not hematoidin (*103*):

> Cholochromic acid differs from hæmatodine by its solubility in alcohol and by crystallizing in (clino ?) rhombic octahedra, not rhombic plates. Rotten bile, and bile treated by the proceeding of Berzelius for obtaining cholochrome have both a dark-pink colour, and chloroform extracts from the former some coloured acid.

Thudichum classified gallstones into seven series and provided examples from the literature in each series (*103*):

> *Classification of Gall-stones.*
>
> *First Series*.–Pellucid or pure cholesterine calculi.
> *Second Series*.–Mixed calculi, with prevalence of cholesterine.
> *Third Series*.–Calculi with prevalence of cholochrome.
> *Fourth Series*.–Calculi with prevalence of modified cholochrome.
> *Fifth Series*.–Gall-stones with prevalence of bile acids.
> *Sixth Series*.–Gall-stones with prevalence of fatty acids.
> *Seventh Series*.–Gall-stones with prevalence of carbonate of lime.

At this point he had carried out only a limited investigation into the pigments (*cholochrome*, as he named them collectively) of bile and gallstones, but that was due to change with his 1868 publication (*152*) on the isolation of a red pigment (*Cholephäin*, or bilirubin) from ox gallstones, its conversion into what he called *cholechlorin* (or biliverdin), and his combustion analyses thereof. It was work which followed that of *Städeler* (*135*) by four years.

In 1868, in a paper on bile pigments written in his native German, *Thudichum* published his experimental results on the red pigment of ox gallstones, its isolation

and purification, physical and chemical properties, combustion analysis and its transformation into salts (ammonium, sodium, potassium, silver, barium, calcium, zinc, and lead). He also described the conversion of bilirubin into biliverdin and discussed its chemical and physical properties, combustion analysis, calcium and barium salts.

The isolation of bilirubin [*Thudichum* used *Städeler*'s name for the pigment interchangeably with his own, *Cholephäin*] from gallstones was pursued by an elaborate series of washings, $CHCl_3$ and alcohol digestions, precipitations, *etc*. excluding exposure to air as much as possible in order to remove traces of bile and bile acid components, and to break apart bile pigment salts. The detailed care taken exceeded even *Städeler*'s. Thus, ox gallstones were pulverized (during which one's air passages were protected from the powder by a kerchief), stirred with a bit of hot H_2O (the same way a cook mixes flour for dough) then bathed in hot H_2O with vigorous stirring before being allowed to stand for two days. The water was drained and the solid left behind was thoroughly washed with H_2O before washing and filtering and washing again until the filtrate was clear. The remaining slurry was transferred to a flask where it was digested with a large quantity of alcohol while being heated to remove bile acids and their calcium as well as some fatty acid salts (but rarely cholesterol). The washed powder was then treated with cold dilute HCl, which evolved CO_2 and H_2S. *Thudichum* found it better to let the HCl do its work on the solid without heating. The solid was washed free of HCl with H_2O by decanting, and then it was treated again twice with alcohol to remove traces of any bile acids. After complete exhaustion, the solid was treated with ether and then dried. At this point the powder had a beautiful reddish-yellow color ("Nach dem Trocknen ist das Gallensteinpulver schön rothgelb."), and it was heated in water and acid-free $CHCl_3$. (It may be important to note that *Thudichum*, like *Städeler* before him, took precaution with the $CHCl_3$, which then doubtless lacked the ethanol stabilizer found nowadays in commercial $CHCl_3$, because it commonly became acidic by reaction with air and light while it partially decomposed into HCl and phosgene.) The $CHCl_3$ solution was filtered from the solid, and the residue was again heated in fresh $CHCl_3$ while certain measures were used to avoid losses of $CHCl_3$. Then with evidence of great care, *Thudichum* removed the red $CHCl_3$ solution from the solid by siphoning, presumably to minimize exposure to air, and distilled off most of the $CHCl_3$. This left a red residue with some green spots admixed, which was washed on a filtration funnel with $CHCl_3$ until it was red and no longer co-mixed with green, while the $CHCl_3$ was all the more yellow-red. A little alcohol was added to the dark, nearly black-green colored mother liquor and red, very finely dispersed bilirubin was removed by filtration and washed with alcohol, and crystals easily formed in the alcohol mother liquor. The pigment obtained was a splendid red, of a color similar to that of the HgO obtained by heating its nitrate. Neither absolute alcohol nor ether extracted any impurities, only traces of pigment. Further purification could be approached by careful, repetitious dissolving in $CHCl_3$ and precipitating the concentrated solution by adding absolute alcohol.

Repeated $CHCl_3$ extractions of powdered gallstones, as above, pretty much lost effectiveness in terms of bile pigment extraction. Yet the remaining powder was treated with alcoholic KOH to dissolve pigment and produce a dark red-brown color, and the solution could be filtered away from a voluminous residue of impurities. Acidification of the solution with HCl precipitated voluminous flakes of red pigment, which was filtered as rapidly as possible from considerable green pigment and then taken up into either alcohol or $CHCl_3$ and further processed to purification.

Prior to the use of $CHCl_3$ to extract bilirubin, only a brown modification (*Gallenbraun*, *Biliphäin*, *Cholephäin*) was typically obtained from icteric urine, bile, or gallstones and was, by the time of *Städeler* and *Thudichum*, recognized as *impure* bilirubin, typically containing (calcium) salts. The then purest form of the pigment was red, hence bilirubin or *Cholerythrin*, became available by extractions involving $CHCl_3$, and *Thudichum* examined the crystals in considerable detail (*107*):

> Vor der Entdeckung des Gebrauchs des Chloroform als ein Lösungmittel für diesen Farbstoff hatte man nur braune Modificationen desselben erhalten und Biliphäin oder Cholephäin benannt. Nachdem indessen der rothe Farbstoff vermittelst Chloroform erhalten worden war, nahm man allgemein an, dass die braune Farbe früherer Präparate ein Zeichen ihrer Unreinheit gewesen sei. Der rothe Farbstoff, unter dem Namen Bilirubin oder Cholerythrin, wurde für die einzige Form von reinem Gallenfarbstoff gehalten. 101

Thudichum stated that from his many isolations and purifications he always found two modifications of the pigment that were chemically identical. One was red-brown, the other was red like the color of HgO. Under a microscope the former exhibited numerous microcrystals among many completely formed crystals. Yet, in contrast, bilirubin consisted almost entirely of small amorphous granules, and only when it was precipitated by alcohol did it yield small yellow rhombic prisms. A mixture was precipitated from a saturated $CHCl_3$ solution of *Cholephäin* by added alcohol. The first precipitate (bilirubin) was captured by filtration; with gradual addition of more alcohol a second crop was obtained – half red and amorphous, half little brown crystals. The crystals seldom remained gathered together in husks but could be separated quickly from the remaining suspended bilirubin by washing with alcohol. Thus, the isolated crystals had a dark red-brown color and their surfaces reflected light with a purple, steel-blue luster (*152*):

> . . . In zahlreichen Operationen, welche ich behufs der Isolirung des reinen Gallenfarbstoffs unternahm, erhielt ich stets zwei Modificationen, die sich zwar chemisch gleich verhielten, wovon jedoch die eine rothbraun, die andere rein roth wie Quecksilberoxyd war. Die mikroskopische Untersuchung ergab, dass der dunkelbraunrothe Farbstoff aus zahllosen krystallinischen Partikelchen mit vielen vollständigen Krystallen bestand. Das Bilirubin dagegen bestand beinahe ganz aus kleinen amorphen Körnchen; nur wenn es mit Alkohol gefällt worden war, enthielt es kleine gelbe rhombische Prismen. Liess ich eine Mischung aus einer gesättigten Chloroformlösung des Cholephäins und absoluten Alkohol bestehend, aus der der erste Niederschlag vom Bilirubin durch das Filter entfernt

> worden war, stehen, und setzte ich allmählich mehr Alkohol zu, so erhielt ich allmählich einen zweiten halb rothen und amorphen, halb krystallinischen braunen Niederschlag. Die Krystalle sassen nicht selten in Drusen zusammen. Sie konnten durch Schlämmen mit Alkohol, in dem sie sich schnell absetzten, von dem länger suspendirt bleibenden Bilirubin getrennt werden. 102

By examination under a microscope, the crystals were seen as opaque, thin reddish or red blades that transmitted light, but the most opaque, of which there were few present, sent yellow light to the eye. Their dimensions and shapes were specified.

These *Thudichum* referred to as *Cholephäin*. The smallest bilirubin crystals showed the same shape and yellow color. By careful recrystallization the red modifications could be changed partially into the brown. From which he concluded that crystallized or microcrystalline purple-brown *Cholephäin* or bilirubin is only a different state of aggregation of amorphous red bilirubin or *Cholerythrin*. As a consequence, he regarded *Cholephäin* and bilirubin as chemically identical and cautioned that when one names the pigment, the process for obtaining it should also be specified (*152*):

> Die kleinsten Bilirubin-Krystalle zeigten dieselbe Gestalt und gelbe Farbe. Durch vorsichtiges Umkrystallisiren konnte die rothe Modification stets theilweise in die braune verwandelt werden. Es ist daher klar, dass das krystallisirte oder krystallinsiche purpurbraune Cholephäin oder Biliphäin nur ein anderer Aggregatzustand des amorphen rothen Bilirubins oder Cholerythrins ist. Ich werde daher in der Folge Cholephäin und Bilirubin als chemisch identisch betrachten, füge aber hinzu, dass wenn in der Beschreibung eines Processes der eine oder andere Name gebraucht wird, die dadurch bezeichnete Modification für den Process benutzt worden ist. 103

Thudichum then described a color change (to brown) when the solid orange pigment was exposed to light [apparently another early example of bilirubin photochemistry] in the absence of moisture (but not apparently in the absence of oxygen). The same brown color was obtained by briefly heating the solid pigment in water. The change in color occurred only on the surface of the pigment, but when heated for a longer time, it became thoroughly brown. Thus it would appear that bilirubin had been converted to the *Gallenbraun* from whence it came, though *Thudichum* did not say so.

The solubility of the (orange) pigment was determined, with results more or less coincident with *Städeler*'s: insoluble in H_2O and slightly soluble in boiling absolute alcohol (with yellow coloration). The latter coloration was apparently due to a dispersion of solid because filtration yielded a colorless filtrate and a colored filter paper. It was slightly soluble in ether, somewhat soluble in CS_2 and in benzene, and had its best solubility in $CHCl_3$: 1.7 parts per 1,000 parts $CHCl_3$ to form a beautiful dark red solution, or about the same solubility as seen today with bilirubin. Sunlight (presumably on the $CHCl_3$ solution) produced a brown to black coloration, which *Thudichum* presumed was caused by the [photochemical] formation of HCl gas. Saturation of the solution with HCl gas followed by complete removal

of the $CHCl_3$ and acid by distillation left a mixture of two beautiful green compounds that could not be separated by differential solubility in alcohol (in which both were soluble) but by ether (in which only one was soluble). The bilirubin had been converted entirely into the two new compounds, which were apparently not investigated further[46] (*152*):

> In Wasser ist der Stoff ganz unlöslich, wenig löslich in kochendem absoluten Alkohol, mit gelber Farbe; filtrirt man diese Lösung durch Papier, so bleibt der Farbstoff der ersten Portionen der Lösung an den Papierfasern haften und der Alkohol fliesst beinahe farblos ab. In Aether ist er wenig löslich, etwas löslicher in Schwefelkohlenstoff und in Benzol. Das beste Lösungmittel ist Chloroform, wovon 1000 Theile, 1,7 Theil, 586 Theile daher einen Theil Bilirubin lösen. Die Lösung ist prächtig dunkelroth gefärbt. Die Sonnenstrahlen verfärben diese Lösung zu Braun und Schwarz, wahrscheinlich durch Bildung von Salzsäure. Der Zusatz von wässeriger Salzsäure bringt einen Niederschlag in der Lösung hervor. Leitet man indessen trockenes Salzsäuregas in die Lösung bis zur Sättigung, und destillirt alsdann Chloroform und Säure vollständig ab, so bleibt eine Mischung von zwei prächtig grünen Körpern übrig, die sich nicht durch Alkohol, worin beide löslich sind, wohl aber durch Aether, worin nur einer löslich ist, trennen lassen. Das Bilirubin geht ganz in diese neuen Verbindungen über. 104

Like *Städeler*, *Thudichum* conducted elemental combustion analyses on his purified bilirubin, or *Cholephäin*. The pigment was dried under vacuum at 100°C, then between 120°C and 130°C to constant weight, which made it a little darker. Six combustion analyses were performed, three for carbon and hydrogen (I–III); three for nitrogen (IV–VI) below. *Thudichum* included all of the relevant weighings to four significant figures and qualified the results of III as having come from too small a sample. The nitrogen analyses were conducted in different ways; that of VI came from again repurified pigment. From the combustion data, *Thudichum* calculated an empirical formula (C_9H_6NO) for *Cholephäin*, a name he used interchangeably with bilirubin (*152*):

Vergleich der Empirie und Theorie der Elementar-Zusammensetzung des Cholephäins.

	I.	II.	III.	IV.	V.	VI.	Mittel
Є	66,02	66,41	65,61	–	–	–	66,01
H	5,97	6,13	5,95	–	–	–	6,01
N	–	–	–	9,05	9,49	8,56	9,03
Θ	–	–	–	–	–	–	18,95
							100,00

Diese Zahlen führen zur Formel $Є_9H_6NΘ_2$,[47] deren Theorie mit obigen Thatsachen folgendermassen sich vergleicht:

[46] One might guess that at least one was biliverdin-IXα, possibly contaminated with XIIIα.
[47] N.B. *Thudichum* probably meant "Formel $Є_9H_9NΘ_2$".

Atom	At.-Gew.	Theorie in 100	Mittel der Analysen
C_9	108	66,26	66,01
H_9	9	5,52	6,01
N	14	8,59	9,03
O_2	32	19,63	18,95
	163	100,00	100,00

105

Writing an element's symbol with a bar through it, such as C and O, was a convention introduced by *Alexander Williamson* and *August Kekulé* well before the Karlsruhe Congress (*147–149*). It signified that the correct atomic mass of the element (12 and 16, respectively) was to be used in the formula and not the equivalent mass (6 and 8) introduced by *Berzelius* and *Gmelin* and widely used in the 1800s to give what today are odd-looking formulas, such as HO for water and $HOSO_3$ for sulfuric acid. As the correct atomic masses gained acceptance, "barred" elements disappeared. Here, *Thudichum* was expressing adherence to the decision at Karlsruhe. It is noteworthy that the (correct) atomic weights agreed upon in the famous 1860 chemical congress in Karlsruhe were used, and the formula weight (= 163) corresponding to the empirical formula $C_9H_9NO_2$ was then called an "atomic weight" (*das Atomgewicht*) rather than molecular weight (*Moleculargewicht*) – in recognition that the formula was not necessarily that of the molecule. *Thudichum* believed his analyses gave the correct formula and actual *Atomgewicht* of *Cholephäin* or bilirubin from a long series of noteworthy compounds as well as from several interesting transformations by acids and bases (*152*):

> Dass obige Formel die richtige, und dass 163 das wirkliche Atomgewicht des Cholephäins oder Bilirubins ist, werde ich in dem Folgenden durch eine lange Reihe merkwürdiger Verbindungen, sowie durch mehrer interessante Umwandelungen dieses Stoffes unter dem Einfluss verschiedener Säuren und Alkalien näher beweisen. 106

For the last, *Thudichum* converted bilirubin into its ammonium, sodium, and potassium salts. To obtain the first he treated the pigment with saturated aq. ammonia to form a dark red voluminous mass. A stream of air was passed through, first cold, then heated to 100°C, to drive off the NH_3 and leave behind a greenish-brown lustrous, brittle mass. In order to learn how much NH_3 was combined with the bilirubin, a carefully weighed and dried sample of the pigment (1.8483 g) was saturated in liq. NH_3 to yield a brown-red solid (1.8589 g) after blowing off the NH_3 and drying in a stream of air at 100°C. The difference in weights of the initial and final pigments indicated how much (0.0106 g) NH_3 or oxygen had been absorbed – to yield the hypothetical formula $[C_9H_8(NH_4)NO_2 + H_2O]$ which predicted an increase in weight of 0.41 g. The much smaller experimental difference in weight undoubtedly confirmed the formulas as only empirical but it was too small or too suspect from which to predict a molecular formula based on a different stoichiometry between NH_3 and the pigment. The ammonia adduct of bilirubin was readily soluble in strong alcohol (95%) and insoluble in ether. As was observed previously by others, bilirubin dissolved in aq. or ethanolic KOH or NaOH and could be precipitated

by acid. Or (in alkaline solution) converted to a green pigment, biliverdin, by warming.

More important possibly were *Thudichum*'s preparations of the silver, barium, calcium, zinc, and lead salts of bilirubin – and their combustion analyses. The silver salt was prepared from a neutral ammoniacal solution of *Cholephäin* (prepared by digestion of an excess of *Cholephäin* in aqueous ammonia and precipitated by the addition of $AgNO_3$). The reddish-brown precipitate thus obtained was dried under vacuum over H_2SO_4 in the dark. The solid was analyzed for silver content/residue after combustion, and the data from three analyses, guided by *Thudichum*'s empirical formula for bilirubin ($C_9H_9NO_2$), were found to be consistent with the neutral hydrated formula, $C_9H_{10}AgNO_3$ (*152*):

> Dass Mittel dieser Bestimmungen ist 37,39 p.C. Ag.
>
> Wenn man nun in Betracht nimmt, dass die Analysen des Cholephäins oder Bilirubins zur empirischen Formel $C_9H_9NO_2$ führen, so kann es keinem Zweifel unterliegen, dass die in dem oben beschriebenen Silbersalze enthaltene Menge Silber genau derjenigen entspricht, welche eine neutrale, einfach gewässerte Verbindung von der Formel $C_9H_{10}AgNO_3$ erfordert. Wie anomal . . . auch immer ein Silbersalz mit einem Atom Wasser sein möge, es ist jetzt gewiss, dass die Elementarzusammensetzung und das Molekül des Cholephäins durch die Formel $C_9H_9NO_2$ ausgedrückt wird.
>
> *Vergleich der Theorie und Empirie des im Vacuo getrockneten Silber-Cholephäinats.*

			Gef.			
Symbole	At.-Gew.	In 100 Th.	a.	b.	c.	Mittel
C_9	108	37,30	–	–	–	–
H_{10}	10	3,47	–	–	–	–
Ag	108	37,50	37,63	37,52	37,03	37,39
N	14	–	–	–	–	–
O_3	48	–	–	–	–	–
	288					

107

What *Thudichum* called the basic silver salt was prepared from *Cholephäin* dissolved in aq. NH_3 and precipitated by addition of $AgNO_3$ and HNO_3 (*152*):

> Eine kleine Menge Cholephäin, welche wiederholt durch Lösen in Chloroform und in alkoholischer Kalilösung gereinigt worden, war, wurde in Ammoniak gelöst und mit Silbersalpeter gemischt. Da kein Niederschlag erschien, so wurde mehr Silberlösung zugesetzt und das ganze dann mit Salpetersäure bis beinahe zur Neutralität abegestumpft. Der jetzt erscheinende Niederschlag liess die Flüssigkeit farblos; er wurde mit Wasser gewaschen und in der Leere getrocknet. 108

Combustion analysis for silver predicted the formula $C_9H_7Ag_2NO_2$, with two silver atoms replacing two hydrogen atoms in *Thudichum*'s empirical formula $C_9H_9NO_2$ (*152*):

> Es ist auf diese Weise ermittelt, dass das Cholephäinsilber in freiem Ammoniak löslich ist, und dass wenn diese Lösung bei Gegenwart von überschüssigem Silbersalpeter auf einen gewissen an Neutralität gränzenden Alkalinitätsgrad herabgestimmt wird, das basische Salz niederfällt. Seine Theorie leitet sich aus den über das freie und mit einfach Silber verbundene Cholephäin bekannten Thatsachen her und wird durch die Analysen

bestätigt; seine Formel ist $C_9H_7Ag_2NO_2$. In dieser Verbindung sind daher zwei Wasserstoffatome durch zwei Atome Silber ersetzt. Ich werde später eine analoge Bleiverbindung beschreiben, in welcher zwei Atome Wasserstoff durch ein didynamisches Atom Blei ersetzt sind. Ihre Formel ist $C_9H_7PbNO_2$, und sie ist eine wesentliche theoretische Stütze für die Annahme, dass das oben beschriebene basische Silbersalz eine wirkliche feste Verbindung und nicht nur eine zufällige Mischung sei.

Vergleich der Theorie und Empirie des basischen Cholephäinsilbers.

			Gef.			
Symbole	At.-Gew.	In 100 Th.	a.	b.	c.	Mittel
C_9	108	28,69	–	–	–	–
H_7	7	1,85	–	–	–	–
Ag_2	216	57,29	56,81	56,41	55,86	56,27
N	14	–	–	–	–	–
O_2	32	–	–	–	–	–
	377					

109

The barium salt was also prepared from an aqueous solution of *Cholephäin* in excess NH_3 by precipitating with added $BaCl_2$. The resultant green precipitate, which *Thudichum* designated a neutral barium *Cholephäinate*, was, after drying, combusted. The analysis data, for C, H, and Ba, were determined to be consistent with the formula $C_{18}H_{20}BaN_2O_6$, or $(C_9H_{10}NO_3)_2Ba$ (*152*):

Diese Thatsachen entsprechen den Anforderungen der Theorie einer dem neutralen Silbersalz genau analogen Baryumverbindung, in welcher ein zweidynamisches Atom Baryum, zwei Moleküle Cholephäin durch Ersatz eines Atoms Wasserstoff in jedem derselben zusammenschweisst; ausserdem treten zwei Moleküle Wasser in die Verbindung ein.

Vergleich der Theorie und Empirie des Baryumcholephäinats,
$C_{18}H_{20}BaN_2O_6$.

			Gef.		
Symbole	At.-Gew.	In 100 Th.	a.	b.	c.
C_{18}	216	43,46	–	–	44,58
H_{20}	20	4,02	–	–	3,98
Ba	137	27,56	27,56	27,55	–
N_2	28	–	–	–	–
O_6	96	–	–	–	–
	497				

110

A somewhat more complicated formula was derived for the half-acid barium *Cholephäinat* (or *Sesquicholiphäinat*) that arose by precipitation from digesting a completely neutralized aqueous solution of $BaCl_2$ with excess *Cholephäin*. The precipitate was washed with H_2O, then digested in alcohol, heated, and washed until the alcohol was colorless. The brown-red product, after powdering and drying, had a dark brown surface. Combustion analysis indicated three molecules of *Cholephäin* to one of barium, corresponding to the formula $C_{27}H_{29}BaN_3O_8$ (*152*):

In dieser Analyse zersprang die Röhre am Ende der Operation, als das Kali in die Sicherheitsblase des Apparats zurückstieg, so dass das Residuum an Kohlensäure und

Wasser nicht ausgesogen werden konnte. Uebrigens zeigen diese Analysen ganz klar, dass in diesem Cholephäinate ein Atom Baryum mit drei Molekülen Cholephäin verbunden ist.

Wenn wir zu dem oben beschriebenen zwiefach gewässerten neutralen Baryumcholephäinat ein Molekül Cholephäin hinzufügen, wie hier

1	Baryum-Cholephäinat,	$\text{C}_{18}\text{H}_{29}\text{BaN}_2\text{O}_6$	=	497	At.	Gew.
1	Cholephäin,	$\text{C}_9\text{H}_9\quad\text{NO}_2$	=	163	At.	Gew.
	so erhalten wir	$\text{C}_{27}\text{H}_{29}\text{BaN}_3\text{O}_8$	=	660	At.	Gew.

Die Analysen der oben beschriebenen Verbindung entsprechen nun dieser Theorie ganz vollständig.

			Gef.				
Symbole	At.-Gew.	In 100 Th.	a.	b.	c.	d.	Mittel
C_{27}	324	49,09	–	–	51,50	49,76	50,63
H_{29}	29	4,39	–	–	4,65	4,09	4,37
Ba	137	20,75	20,66	20,60	–	–	20,66
N_3	42	–	–	–	–		–
O_8	128	–	–	–	–		–
	660						

111

In like manner, the neutral and half-acid calcium *Cholephäinats* were prepared and analyzed to indicate probable formulas $C_{18}H_{20}CaN_2O_6$ for the former and $C_{27}H_{29}CaN_3O_8$ for the latter (*152*):

Die von diesen Daten abgeleitete Formel führt zu einem neutralen Calciumcholephäinat $\text{C}_{18}\text{H}_{20}\text{CaN}_2\text{O}_6$, welches in jeder Beziehung der oben beschriebenen Baryumverbindung analog ist. Auch in ihm müssen wir die Existenz von 2 Mol. Wasser annehmen, welche durch eine Temperatur von 100° nicht ausgetrieben werden.

Folgender Vergleich der Theorie dieser Verbindung mit den analytischen Daten wird die Richtigkeit dieses Schlusses leicht anschaulich machen.

	Theorie		Gef.			
Symbole	At.-Gew.	In 100 Th.	a.	b.	c.	d.
C_{18}	216	54,00	–	–	–	52,35
H_{20}	20	5	–	–	–	5,04
Ca	40	10	9,63	9,88	9,92	–
N_2	28	–	–	–	–	
O_6	96	–	–	–	–	
	400					

	Gef.				Mittel
	e.	f.	g.	h.	
C	–	–	54,96	54,26	53,86
H	–	–	5,03	4,65	4,9
Ca	11,02	10,44	–	–	10,17

. . . Die Verbindung ist demnach dem bereits beschriebenen halbsauren Baryumcholephäinat analog und hat die Formel $C_{27}H_{29}CaN_3O_8$. Mit dieser Ansicht stimmen die Resultate der Analysen wie folgt.

	Theorie		Experimente.		
	der Atome	p.C.	a.	b.	c.
C_{27}	324	57,54	–	–	60,37
H_{20}	29	5,15	–	–	5,74
Ca	40	7,1	7,03	6,79	–
N_3	42	–	–	–	–
O_8	128	–	–	–	–
	563				

Durch die nachfolgende Zusammenstellung werden die Unterschiede in der Zusammensetzung des neutralen Calciumcholephäinats auf der einen und des halbsauren auf der anderen Seite sehr deutlich.

Neutrales Calcium-Cholephäinat, $C_{18}H_{20}CaN_2O_6$. At.-Gew. = 400.		
	Theorie	Gef.
C	54	53,86
H	5	4,9
Ca	10	10,17

Halbsaures Calcium-Cholephäinat, $C_{27}H_{29}CaN_3O_8$. At.-Gew. = 563			
	Theorie	Gef.	
C	57,54	60,37	
H	5,15	5,74	
Ca	7,1	6,91	112

The analytical data for these calcium salts were compared with *Städeler*'s data for his calcium salt of bilirubin from human gallstones. At issue for *Thudichum* was the interpretation of *Städeler*'s 9.1% CaO datum and his assumption that he was analyzing the neutral calcium salt. *Thudichum* reminds us of *Städeler*'s empirical formula ($C_{18}H_9NO_4$) published in 1861 in *Frerichs*' 2nd edition (*120*) of his famous *Klinik der Leberkrankheiten* (which did not involve analysis data for a calcium salt) and his subsequent formulas, $C_{32}H_{18}N_2O_6$ for free bilirubin and $C_{32}H_{17}CaN_2O_6$ for its calcium salt, published in 1864 (*135*). The formulas, irrespective of their being derived using the old notation [of atomic weights for O (=8) and C (=6)], were said to be incorrect by *Thudichum* because they had been derived for the neutral calcium salt of bilirubin rather than the half acid salt, which *Thudichum* showed fit the %Ca datum better. Which thus meant that *Städeler*'s formulas for biliverdin, *Biliprasin*, *Bilifuscin* (bilifuscin), and *Bilihumin* (bilihumin) would by necessity be incorrect (*152*):

> In seinen Untersuchungen über den Farbstoff menschlicher Gallensteine stellte Städeler eine Calciumverbindung des Bilirubins dar, welche ihm bei der Analyse 9,1 p.C. Calciumoxyd ergab. Von der Annahme ausgehend, dass diese Verbindung ein normales Neutralsalz sei, bestimmte er nach ihr das Atomgewicht des Bilirubins. Er verwarf demnach seine früheren Analysen des krystallisirten Cholephäins, wie sie in Frerich's Klinik der Leberkrankheiten mitgetheilt waren, sowie auch die empirische Formel $C_{18}H_9NO_4$ und substituirte $C_{32}H_{18}N_2O_6$ als die Formel des freien Bilirubins, und $C_{32}H_{17}CaN_2O_6$ als die des Calciumbilirubats (die vorstehenden drei Formeln sind in der alten Notationsweise gegeben). Da nun diese Formeln nur durch eine, hierfür ungenügende, Atomgewichtsbestimmung

durch Kalkgewicht unterstützt sind, auf der anderen Seite aber alle Analysen Städeler's über das Bilirubin and Cholephäin mit meinen Resultaten in vollständigem Einklang gebracht werden können, so kann ich nicht zögern, die Formeln, welche dieser Forscher für Bilirubin und Calciumbilirubat gegeben hat, für irrthümlich zu erklären.

Das von Städeler analysirte Bilirubat war offenbar das halbsaure Salz.

	Theorie von $C_{27}H_{29}CaN_2O_6$ erfordert	Städeler fand
CaO	9,94 p.C.	9,1 p.C.
Ca	7,1 „	6,5 „

Mit der Formel Städeler's für das Bilirubin fallen die Formeln aller anderen von ihm beschriebenen Derivate des Gallenfarbstoffs, namentlich des Biliverdins, Biliprasins, Bilifusins und Bilihumins. 113

Thudichum also prepared zinc- and lead-*Cholephäinat* and carried out combustion analyses of each. The red-brown zinc salt, from $ZnSO_4$, was reddish-brown; the lead salt was from $Pb(OAc)_2$. Analyses for zinc, determined as ZnO, predicted a half-acid salt, $C_{27}H_{29}ZnN_3O_8$, composed of one molecule of neutral zinc *Cholephäinat* ($C_{18}H_{18}ZnN_2O_4$), one molecule of *Cholephäin* ($C_9H_9NO_2$), and two of H_2O ($2\times H_2O$). In comparison a neutral salt of formula $C_{18}H_{10}ZnN_2O_6$ (M.W. 389) would give 16.70% Zn (*152*):

Nach diesen Daten ist es klar, dass das Zinksalz den halbsauren Salzen des Baryums und Calciums analog zusammengsetzt ist, wie folgt.

1	neutrales Zinkcholephäinat	C_{18}	$H_{16}ZnN_2$	O_4
2	Wasser		H_4	O_2
1	Cholephäin	C_9	H_9 N	O_2
1	Mol. halbsaures Zn Choleph.....	C_{27}	$H_{29}ZnN_3$	O_8

Mit dieser Auffassung harmoniren die Resultate der Analysen wie folgt.

	Theorie		Gef.	
	der Atome	p.C.	a.	b.
C_{27}	324	–	–	–
H_{29}	29	–	–	–
Zn	65	11,05	12,03	11,30
N_3	42	–	–	–
O_8	128	–	–	–
	588			

Ein neutrales Cholephäinat von der Formel $C_{18}H_{16}ZnN_2O_4$ Mol.-Gew. = 389 hätte 16,70 p.C. Zn erfordert. 114

And from the lead salt analyses, with the lead being analyzed as lead oxide, as a basic *Cholephäinat* or as *Cholephäin*, the formula $C_9H_7PbNO_2$ was correlated, in which two hydrogens are replaced by one divalent lead. A formula corresponding to the di-silver salt of *Cholephäin* ($C_9H_7Ag_2NO_2$) seen above (*152*):

Diese Verbindung kann als ein basisches Cholephäinat oder als Cholephäin aufgefasst werden, in welchem zwei Atome Wasserstoff durch ein zweidynamisches Atom Blei ersetzt sind.

	Theorie		Gef.	
	der Atome	p.C.	a.	b.
C_9	108	29,34	–	–
H_7	7	1,9	–	–
Pb	207	56,25	58,38	57,91
N	14	–	–	–
O_2	32	–	–	–
	368			

Diese Verbindung entspricht dem basischen Silbercholephäinat oder zweifach Silbercholephäin $C_9H_7Ag_2NO_2$, welches oben näher beschrieben worden ist. 115

Thudichum also prepared biliverdin, which he had previously named *cholechloine*, studied its chemical and physical properties, conducted combustion analysis (from which he derived an empirical formula), and prepared calcium and barium salts for combustion analyses. Biliverdin was thus dissolved in aq. KOH and exposed to air until it was completely green in thin films. Since the reaction could take two or three weeks, in order to accelerate it the solution was heated while air was introduced. Addition of HCl precipitated large green flakes of biliverdin. The precipitate was purified by washing through and through with water on a filter. In yet another synthesis to convert bilirubin to biliverdin, the first was heated with an alkaline solution of copper and potassium acetate. Cuprous oxide precipitated and after removal by filtration, the filtrate was acidified with HCl to precipitate biliverdin. A part of the copper was bound in a characteristic/peculiar way, however, to part of the biliverdin and the free pigment could not be easily released from it.

Under moist conditions, biliverdin was a voluminous mass of a magnificent dark green color. After drying it shrank to a lustrous brittle mass with a completely black color. Its powder was very dark green, and it had not yet been possible at the time to obtain it in a crystalline state. It was completely insoluble in H_2O, ether, and $CHCl_3$. In a moistened condition it was easily soluble in alcohol, but when dry it was much less soluble. It was more soluble in hot alcohol than in cold. By heating its conc. alcohol solution for a long while, it appeared to be transformed and became much less soluble. It dissolved in HCl with a green color, and the solution gave an amorphous green precipitate with $PtCl_4$ and with corrosive sublimate of mercury ($HgCl_2$). After dissolving biliverdin in aq. KOH, when H_2SO_4 was added the color gradually changed to brown-green from the original green.

Thudichum described (*152*) a wide array of reactions but, absent a knowledge of the pigments' structures, they offered no information other than possibly being characteristic of that given pigment. Nor did the reactions reveal much in the way of structural information, for in 1868 the concept of organic structure was in its infancy. *Thudichum*'s reactions consisted of the following: (i) Zn metal added to a solution of biliverdin in hydrochloric acid changed the color from green to brown-red; (ii) Na(Hg) added to an alkaline solution changed the color from green to

reddish-brown, which then changed to green upon introduction of air and also precipitated a brown flocculent mass upon addition of HCl – experiments that showed that reduction of biliverdin by the usual methods did not convert it back to bilirubin. (iii) Reaction with I_2 led to a greenish-black resin, while reaction with Cl_2 in H_2O converted biliverdin to dirty yellow-colored flakes that were insoluble in H_2O and ether but readily soluble in alcohol. But when a saturated alcohol solution of biliverdin was subjected to a small blast of Cl_2 gas, the solution went colorless immediately and yielded chlorine-containing whitish-yellow flakes that were insoluble in H_2O and melted to a reddish-yellow mass upon gentle heating. A yellowish white resin containing several chlorine-containing compounds was obtained by treating biliverdin with hydrochloric acid, followed by gradual addition of $KClO_3$ during warming. Included were one soluble in $CHCl_3$, two in alcohol, but none in ether. (iv) When an alcoholic solution of biliverdin was heated with pure, moist Ag_2O, the pigment was converted to a purple-colored compound, *Bilipurpurin* (bilipurpurin). For the most part bilipurpurin remained insoluble and bound to Ag_2O, but it dissolved in ammonia to yield a green color and an excess of Ag_2O. Hydrochloric acid or any one of several other acids freed up the bilipurpurin, which remained as water-insoluble but easily alcohol-soluble, brownish-red flakes and masses following evaporation of the alcohol and HCl and extraction of NH_4Cl with H_2O.

The properties of the pigment facilitated detection of even small amounts. Thus, to bilirubin or biliverdin dissolved in aq. ammonia was carefully added a little $AgNO_3$ such that all of the excess silver was dissolved. The solution became or remained green after heating and was filtered to remove reduced silver; then alcohol was added followed by an acid, such as HCl, whence the green solution assumed a purple color.

If Ag_2O were left for a longer time in contact with an alcoholic solution of biliverdin, the reaction went over to the formation of bilipurpurin, and the greenish-black solution after treating with ammonia and precipitating the silver by H_2S, gave a clear yellow filtrate. After the alcohol had been removed, a yellow precipitate remained from which, after washing with H_2O and recrystallization from alcohol, left crystals of sulfur to separate upon longer standing. The mother liquor, freed from sulfur, remained yellow and was somewhat soluble in H_2O. Though the entire operation resulted in a significant loss of the original material, the ultimate result was always a yellow-brown compound that appeared as spherical crystalline granules that were easily soluble in alcohol, poorly soluble in H_2O, insoluble in ether, but dissolved in aq. ammonia or KOH, and were precipitated by HNO_3 or HCl. *Thudichum* designated the product *biliflavin*. When an alcoholic solution of biliverdin was heated with HgO alone, or after addition of ammonia, no transformation was noticed. If PbO_2 were used instead of HgO, the biliverdin was transformed into a brown material, or was insoluble, partly perhaps as a lead salt. When heated with aqueous peroxide and ammonia, the biliverdin solution assumed a brownish-red color. When an alcoholic solution was used, it became light yellow and developed the odor of aldehyde or ethyl acetate. Thus, biliverdin was apparently transformed into biliflavin by Ag_2O.

Thudichum also noted (*152*) that biliverdin underwent the *Gmelin* color change reaction following addition of conc. HNO_3 to an alcohol solution of the pigment: first blue, then violet, next red, finally yellow after standing for a long time or heating. When no alcohol was present, the pigment precipitated, and its blue and red colors appeared much less intense to the eye. Before the pigment underwent conversion to the yellow substance, it dissolved in HNO_3 and the transformation proceeded to a maximum. If one accelerated the reaction by heating, then considerable HNO_2 was formed, and yellow flakes of a nitro compound separated from the solution upon addition of H_2O. The aqueous acidic liquor contained a fixed acid that formed a crystalline salt with Ag_2O.

He discussed the solubility of biliverdin in alkali and various salts that he prepared, including those of Ca^{+2}, Ba^{+2}, Pb^{+2}, Cu^{+2}, and Hg^{+2}, finding the pigment to be soluble in aq. potash, NaOH, and NH_4OH. On standing or heating, it became brownish and the precipitate that formed had lost much of its solubility in alcohol. Calcium and barium salts caused no precipitation from aqueous ammonia solutions of biliverdin, but addition of $Ca(OH)_2$ or $Ba(OH)_2$ to an alcohol solution of biliverdin produced green, water-soluble precipitates that were subjected to elemental combustion analysis, as was purified biliverdin itself.

Thus an alkaline solution of the bilirubin analyzed previously as numbers III and IV (*152*) was oxidized by air, over time and without heating to produce the biliverdin used for analyses (a) and (b), below. For analysis (c), the biliverdin was purified by dissolving in hot alcohol then cooling to precipitate, followed by drying the precipitate under vacuum. For analyses (d) and (e), the pigment remaining from analysis (c) was dissolved in hot alcohol and filtered before cooling. The results of *Thudichum*'s combustion analyses of biliverdin and a comparison with those from his bilirubin analyses are shown in the following (*152*):

Zusammenstellung und Mittel der Analysen.

	a.	b.	c.	d.	e.	Mittel
C	–	63,08	62,09	–	62,14	62,43
H	–	6,25	6,12	–	6,00	6,13
N	9,32	–	–	9,36	–	9,34
O	–	–	–	–	–	22,10
						100,00

Vergleicht man diese Befunde mit den das Bilirubin betreffenden Thatsachen,

	Bilirubin		Biliverdin	
	Theorie in 100	Mittel der Analyse		Theorie
C	66,26	66,01	62,43	63,57
H	5,52	6,01	6,13	5,96
N	8,59	9,03	9,34	9,27
O	19,63	18,95	22,10	21,20

116

In comparing the bilirubin and biliverdin data it may be noted that the %C has dropped from the former to the latter, with a small increase in %H and larger increases in the %N and %O. How was this explained? *Thudichum* cited *Heintz*'s

theory (*97*) that biliverdin is an oxide of *Cholephäin* (though he did not use the latter word), and that *Städeler* proposed (*135*) that biliverdin was a hydrated oxide. He calculated, on the basis of his formulas, that if biliverdin were a simple oxide, then one would have $C_9H_9NO_2 + O \rightarrow C_9H_9NO_3$, which could compute as 60.33% C, 5.02% H, and 7.82% N – or rather far from the percentages shown in the small tables above and thus completely contradicting the "oxide" theory. *Städeler*'s hypothesis, too, which might be formulated $C_9H_9NO_2 + O + H_2O \rightarrow C_9H_{11}NO_3$, falls even shorter with its computed 54% C and a corresponding diminution in %H and %N. *Thudichum* calculated 62.81% H, and 8.13% N for a biliverdin composed of two molecules of *Cholephäin* and one of H_2O ($2 \times C_9H_9NO_2 \rightarrow C_{18}H_{20}N_2O_5$). While the %C is an acceptable match, the computed percentage for N falls rather short, and the %H somewhat less short. Thus biliverdin could not be an oxide or hydrate or both (*152*):

> . . . so findet man, dass das Bilirubin, um in Biliverdin überzugehen, viel Kohle verloren, ein wenig Wasserstoff gewonnen, seinen Gehalt an Stickstoff etwas vergrössert und den Sauerstoffgehalt beinahe so viel vermehrt hat, als den Kohlenstoff vermindert. Nach der Theorie von Heintz war das Biliverdin ein Oxyd des Cholephäins gewesen, nach der Hypothese von Städeler ein gewässertes Oxyd. Wäre des Biliverdin ein einfaches Oxyd des Bilirubins ($\text{C}_9H_9N\text{O}_2 + \text{O} =$)C_9H_9NO_3, so würde es 60,33 p.C. C, 5,02 p.C. H und 7,82 p.C. N erfordern. Ein Vergleich dieser Zahlen mit denen, welche die Analysen des Biliverdins ergaben, verneinen indessen die Ansicht, dass das Biliverdin ein Oxyd des Bilirubins sei, vollständig. Die Hypothese von Städeler ist noch viel weniger anwendbar, da die Formel $\text{C}_9H_9N\text{O}_2 + \text{O} + H_2\text{O}$, 54 p.C. C und eine entsprechend verminderte Menge von H und N verlangt. Bestände das Biliverdin aus zwei Molekülen Cholephäin, verbunden mit einem Atom Wasser oder $2(\text{C}_9H_9N\text{O}_2) + H_2\text{O} = \text{C}_{18}H_{20}N_2\text{O}_5$, so wären 62,81 p.C., 5,81 p.C. H und 8,13 p.C. N erforderlich. Selbst wenn die gefundene Kohlenstoffmenge diese Annahme erlaubte, so würde doch der H- und N-Beträgt dieselbe vollständig verneinen. Das Biliverdin ist weder eine Oxyd, noch ein Oxydhydrat, noch ein Hydrat des Bilirubins. 117

Reassessing his experimental combustion analysis data for biliverdin, *Thudichum* computed a C:H:N ratio equal to 7.8:9.2:1, to give the formula $C_8H_9NO_2$, or one carbon atom fewer than in his bilirubin formula (*152*):

> Bei der Berechnung der Formel führen die Durchschnitt der Elementaranalysen zu den Verhältnissen
>
> $$N_1 : H_{9,2} : \text{C}_{7,8}.$$
>
> Diess giebt die Formel C_8H_9NO_2 als die des Biliverdins. Die Analysen stimmen mit dieser Theorie wie folgt.

Atome	At.-Gew.	In 100 Th	Mittel der Empirie
C_8	96	63,57	62,43
H_9	9	5,96	6,13
N	14	9,27	9,34
O_2	32	21,20	22,10
	151	100,00	100,00

118

How can bilirubin be converted to biliverdin? This is much easier using oxygen from the air than it is to understand based on *Thudichum*'s formulas. Yet *Thudichum* proposed that a molecule of bilirubin combined with a molecule of oxygen to form a

molecule of biliverdin and expel a molecule of CO_2: $C_9H_9NO_2 + O_2 \rightarrow C_8H_9NO_2 + CO_2$. Then he concluded his long and important 1868 publication with combustion analyses of the calcium and barium salts of biliverdin. The analysis data for the calcium salt are shown below (*152*):

Zusammenstellung der Analysen und Vergleich mit der Theorie des Zweifach-Calcium-Neunfach-Biliverdin.

	Theorie		Analysen				
	der Atome	p.C.	a.	b.	c.	d.	Mittel
C_{72}	864	60,20	–	–	61,33	63,06	62,19
H_{77}	77	5,36	–	–	5,68	5,8	5,74
Ca_2	80	5,56	5,52	5,77	–	–	5,64
N_9	126	–	–	–	–	–	–
O_{18}	288	–	–	–	–	–	–
	1435						

119

Thudichum found that the %Ca fit best for a formula with a ratio nine biliverdins to two of Ca: $C_{72}H_{77}Ca_2N_9O_{18}$, which was clearly an attempt to fit the combustion data to some sort of formula. But it was apparently one compound and not a mixture with free biliverdin because no biliverdin could be washed out into alcohol (*152*):

> Aus der Menge des gefundenen Calciums berechnet sich das Atomgewicht 709, welches aber offenbar verdoppelt werden muss, damit das Atomgewicht des Biliverdins im Residuum mit einfachen Quotienten aufgehe. $\frac{1418-80+4}{9} = 149$, welches von dem direct gefundenen Atomgewicht des Biliverdins 151, so gut wie nicht verschieden ist. Die Verbindung besteht daher aus 9 At. Biliverdin und 2 At. Calcium. Wären Gründe vorhanden, den Austritt von 1 At. Wasser aus der Verbindung anzunehmen, so erhielte man eine absolute Uebereinstimmung der Theorie mit den Analysen. . . .
>
> Dass dieser Körper eine Verbindung und nicht etwa eine Mischung von einer Kalkverbindung mit freiem Biliverdin ist, geht unter anderen aus dem Umstande hervor, dass er in Alkohol unlöslich ist. Enthielte er freies Biliverdin, so müsste Alkohol dasselbe leicht ausziehen. 120

Analysis of the barium salt and correlation with a formula proved to be somewhat less complicated. The data fit the formula of a half-acid barium *Biliverdat* salt, $C_{24}H_{27}BaN_3O_7$, according to the following reckoning (*152*):

> Das Mittel des gefundenen Baryums, 22,41 p.C., führt zum Atomgewicht 611, welches durch die Operation $\frac{611-137+2}{151} = 3$ und ein Residuum von 23 führt, das man vielleicht als ein Atom Wasser unterbringen darf. Eine kleine Stütze für diese Annahme erhält man aus der Zusammensetzung der Barytsalze des Cholephäins, die alle Wasser, aber auf ein Atom Baryum zwei Moleküle desselben enthalten. Nach dieser Annahme ist das Biliverdat des Baryums ein halbsaures, einfach gewässertes, bestehend aus
>
> | | | | |
> |---|---|---|---|
> | 1 | Mol. | neutrales Biliverdat . . . . | $C_{16}H_{16}BaN_2O_4$ |
> | 1 | Mol. | Biliverdin . . . . . | $C_8H_9 \quad N O_2$ |
> | 1 | Mol. | Wasser . . . . . | $H_2 \quad O$ |
> | 1 | Mol. | halbsaures Ba-Biliverdat | $C_{24}H_{27}BaN_3O_7$ |
>
> 121

	Theorie		Experimente			
	der Atome	p.C.	a.	b.	c.	d.
C_{24}	288	47,52	–	–	49,39	48,20
H_{27}	27	4,45	–	–	4,43	4,34
Ba	137	22,60	22,15	22,67	–	–
N_3	42	6,93	–	–	–	–
O_7	112	18,50	–	–	–	–
	606	100,00				

However, a formula $C_{24}H_{25}BaN_3O_6$ would also fit the combustion data, according to *Thudichum*, if a molecule of H_2O were left out of the calculation. This gives a better fit to the %C but a slightly less good fit for the %Ba (*152*):

> Lässt man das eine Atom Wasser aus der Berechnung weg, so stimmt die Theorie des Kohlenstoffs besser mit der Erfahrung, aber die des Baryums weniger gut.

			Mittel
C_{24}	288	48,97	48,79
H_{25}	25	4,25	4,38
Ba	137	23,29	22,41
N_3	42	7,14	–
O_6	96	–	–
	588		

122

Although *Thudichum* concluded his major work on bile pigments in 1868, he continued investigating reactions of bilirubin and biliverdin into the mid-1870s, especially reactions involving halogens, reduction to hydrobilirubin and the coloring matter of urine (*158–160*). In his erstwhile last work on bile pigments, in 1876, he published on some reactions of biliverdin (*159*, *160*), and in the same year he published (*161*, *162*) a critique and rebuke of *Maly*'s published work of 1868–1876. As we shall see in the latter, he took issue with *Maly's* combustion analysis of the product of bilirubin bromination, other reactions of bilirubin (including reductions by Na(Hg) and Zn and the relationship of the product(s) to urobilin from urine), the formulas for bilirubin and biliverdin, *etc*. in 41 itemized points. Though he published on bile in 1881 (*101*), in a chapter in his edited book addressing mainly bile acids a cursory examination of the pigments from pig gallstones was provided (*101*). Seemingly from the late 1870s and forward *Thudichum* had reoriented and refocused his typical scientific rigor and his efforts to the chemical constituents of the brain (*163*), seminal work of considerable importance and for which he holds a well-deserved reputation. Never one to pass up an opportunity to bring correction to perceived errant science in the bile pigment field, near the end of his life his zest for polemics had not waned, as we shall see at the end of Section 2.10.

2.9.3 Richard L. Maly and Bilirubin

Maly[48] accomplished his major work on bile pigments in Austria between the early 1860s and mid-1870s. His first reading on the subject of the chemical nature of bile pigments was very brief and appeared in part in early 1864 (*150*) as a preliminary communication (*Vorläufige Mittheilungen über die chemische Natur der Gallenfarbstoffe*). It was submitted in longer form from Graz in April 1864 for publication (*164*) in the 1864 *Annalen der Chemie und Physiologie* (now *Liebig's Annalen der Chemie*) and read before the Academy (*165*) at its May 12, 1864 meeting (*vorgelegt in derselben Sitzung, nr. XIII*) but not published in those proceedings. At the time, *Maly* held Dr. med. and Dr. phil. degrees and was stationed at the Universität Graz, where he was *Assistent der Physiologie*. The early work of *Maly* is interesting in that he postulated a surprising new relationship between bilirubin and biliverdin, which, *inter alia*, subsequently became a contentious issue between him and *Thudichum*.

Maly wrote that crystallized *Cholepyrrhin* (*Biliphäin*) – in 1864, while in Graz, he either did not know or did not subscribe to *Städeler*'s new term "bilirubin" – behaved like an amide toward alkali because it released NH_3 and yielded a yellow or green pigment (*150*, *164*):

> . . . Dieses verhält sich zu Alkalien wie ein Amid, d. h. entwickelt damit Ammoniak, während der Rest sich mit den Basen zu gelben oder grünen salzartigen Körpern vereint.
>
> Alles Cholepyrrhin war zu den angestellten Versuchen zweimal umkrystallisirt; von ihnen theile ich vorderhand mit Ausschluss von Analysen Folgendes mit:
>
> Alkoholische oder wässerige Kalilösung entwickelt aus Cholepyrrhin schon bei gewöhnlicher Temperatur Ammoniak; die Flüssigkeit färbe sich für kurze Zeit roth und wird dann grüngelb.
>
> Eben so wirkt Natronlauge. 123

That is, heating *Cholepyrrhin* in alcoholic or aq. KOH or NaOH at the usual temperature released NH_3 and left briefly a red solution that turned green-yellow. Heating with $Ba(OH)_2$ or $Ca(OH)_2$ produced NH_3 and yielded Ba^{+2} or Ca^{+2} salts.

The *Cholepyrrhin* used had been isolated as a red-yellow pigment from human bile using the $CHCl_3$ extraction method of *Valentiner* (*107*, *108*) and *Brücke* (*109*) and purified according to the latter by crystallization. *Maly* was apparently unaware that *Städeler* (*135*) and *Thudichum* (*102*) had to use more heroic methods to free

[48] *Richard L. Maly* was born on June 28, 1839 in Graz and died on March 23, 1891 in Prague. He studied pharmacy and medicine at the University of Vienna and in 1864 was awarded the Dr. med. degree. In the same year he habilitated at the University of Graz for surgical science preparation. In 1866 he was promoted to Professor of Medicine-Surgery at the Lehranstalt Olmütz, then in 1869 to Professor of Physiological Chemistry at the University of Innsbruck and in 1875 to Professor of General Chemistry at the Technische Hochschule in Graz. Not one to remain long in one location, in 1886. *Maly* accepted his final professorship (in general chemistry) at the Deutsche Universität in Prague (currently Charles University). His interest in natural products appears to have migrated from work on abietic acid in the mid-1860s to bilirubin, and much of the latter work was presented at the *Sitzungberichte der kaiserlichen Akademie der Wissenschaften in Wien*.

the gallstone-derived purer bilirubin from its calcium and other occluded salts. From his experiments, he concluded that biliverdin is an acid and *Cholepyrrhin* is its amide, which he named *Biliverdinamid* (biliverdin amide) (*150*), or an ammonium salt (*164*):

> Das Biliverdin ist eine Säure, das Cholepyrrhin ihr Amid (Biliverdinamid), ersteres gehört dem Wasser – letzteres dem Ammoniaktyp an; oder Biliverdin und Cholepyrrhin verhalten sich wie Kohlensäure und Harnstoff. 124

This contention was reinforced by an experiment in which *Cholepyrrhin* was heated in a mixture of $CHCl_3$-acetic acid and thereby converted to a green color. After washing with H_2O (to extract acetic acid) and evaporating the aqueous layer, a white substance containing what was said by *Maly* to be ammonium acetate was left behind; whereas, evaporation of the $CHCl_3$ layer, washed free of acetic acid, left a black-green residue of what was said by *Maly* to be pure biliverdin (*164*):

> Der Inhalt eines solchen Rohrs wurde in Wasser gegossen; unten sammelte sich die dunkelgrüne Chloroformschichte, während das Wasser den Eisessig aufnahm. Erstere Schichte wurde so lange mit Wasser gewaschen, als dieses sauer abfloss. Dann vereinigte man die wässerigen Flüssigkeiten und brachte sie im Wasserbade zur Trockne. Der Rückstand in concentrischen weissen Ringen enthielt essigsaures Ammonium; es war also ein Theil des Stickstoffs im Cholepyrrhin durch die Einwirkung des Eisessigs in Form von Ammoniak abgespalten. Die mit Wasser gewaschene und von der Essigsäure befreite Chloroformschichte gab, nachdem das Lösungsmittel abgedunstet war, einen dunkelfast schwarzgrünen Rückstand von reinem Biliverdin. 125

Maly found that HCl and tartaric acid gave essentially the same reaction with *Cholepyrrhin*, and from the collective data, he became convinced that *Cholepyrrhin* was an amide (but was not an ammonium salt) (*164*):

> Diese und die vorigen Reactionen lassen unverkennbar das Cholepyrrhin als ein Amid erscheinen (ein Ammoniumsalz hätte zur Spaltung wohl keiner so lange dauernden Einwirkung gebraucht), das sowohl, wie der Character der Amide mit sich bringt, durch Alkalien, als durch Säuren gespalten wird, in die entsprechende Säure – hier Biliverdin – und in den Rest NH_3, der im ersten Falle entweicht, im zweiten als einfaches Ammonium salz sich vorfindet.
>
> Das Biliverdin ist eine Säure, des Cholepyrrhin ihr Amid (Biliverdinamid). Ersteres gehört dem Wasser-, letzteres dem Ammoniaktypus an, oder sie verhalten sich wie Kohlensäure und Harnstoff. 126

The state of knowledge of organic chemistry in 1864 was apparently insufficient to cause one to puzzle that conversion of an amide to its acid might cause a color change from red-yellow to green. Knowledge of organic structure was then only primitive and thus a correlation between a chromophore and its "color" was absent. "Spectroscopy" in the visible region of the spectrum was yet a few years distant in organic or physiological chemistry. And *Maly*'s experimental "conversion" of biliverdin back to *Cholepyrrhin* through the action of NH_3 involved heating what *Maly* called the ammonium salt of the former, which could well have been the ammonium salt. But the *Cholepyrrhin* allegedly formed was at the time identified only by its color and solubility in $CHCl_3$. Again, at the time of this work there was only a sketchy knowledge of organic structure and its relationship to reactivity.

The work, novel though it was, was retracted by *Maly* some four years later (*166*, *167*), with the adventitious NH_3 being attributed to an impure sample of *Cholepyrrhin*; thus, in February 1868, *Maly* would write (*166*):

> Ammoniak und die ätzenden Alkalien lösen das Cholepyrrhin mit braunrother Farbe. Bei Anwendung der letzteren schien mir früher . . . eine Entwicklung von Ammoniak statt zu finden. Dieser Irrthum wurde aber durch eine nicht ganz reine aus Menschengalle erhaltene Substanz hervorgerufen. Den damals daraus gezogenen Schluss nehme ich daher zurück. Gegenwärtig nach viel weitläufigeren Beobachtungen bin ich vielmehr zu der weiter unten durch Belege begründeten Ueberzeugung gelangt, dass bei dem Uebergange von Cholepyrrhin in Biliverdin kein Ammoniak sich abspaltet, und dass in letzterem Körper gleichwie in ersterem noch dieselbe atomistische Menge Stickstoff enthalten ist. 127

Maly's sources of *Cholepyrrhin* were human and ox gallstones, the former of which he recognized were often rich in the calcium salt of the pigment. The latter had little or no cholesterol, and served as a convenient source of purified *Cholepyrrhin*. (In 1868, *Maly* was able to acknowledge that the orange pigment of bile had accumulated three names, from *Berzelius*' *Cholepyrrhin* to *Simon*'s *Biliphäin* to the most recent name: *Städeler*'s *Bilirubin* (bilirubin), given in 1864.) *Maly* conducted C, H elemental combustion analyses of his *Cholepyrrhin*, isolated from both human and ox gallstones, and obtained data very closely coincident with *Städeler*'s (*135*). *Maly* calculated the formula $C_{16}H_{18}N_2O_3$ for it (*166*):

Analyse.

I. 0,2770 Grm. aus Menschengallensteinen gaben bei der Verbrennung 0,681 Grm. $C\!\!\!\!-O_2$ und 0,1545 Grm. $H_2O\!\!\!\!-$.
II. 0,2734 Grm. Cholepyrrhin aus Ochesengallensteinen gaben 0,1532 Grm. Wasser.

Diese giebt in 100 Theilen Substanz:

	I.	II.
Kohlenstoff.....	67,16	–
Wasserstoff.....	6,18	6,22

Diese Zahlen zeigen mit der Berechnung für $C_{16}H_{18}N_2O\!\!\!\!-_3$ und mit den analytischen Mittelzahlen von Städeler:

	Bez. für $C_{16}H_{18}N_2O_3$	Mittel von Städeler (i.e.)
Kohlenstoff.....	67,13	67,13
Wasserstoff.....	6,29	6,19

> eine so grosse Uebereinstimmung, dass ich dadurch über die Zusammensetzung dieses Körpers völlig versichert, nicht weiter Material zu Analysen opfern wollte. 128

Maly reconfirmed some of the then recent earlier observations of the pigment's solubility properties: a little soluble in benzene, insignificantly soluble in petroleum ether, somewhat more soluble in hot amyl alcohol, in fatty oil, and glycerin. He noted that it dissolved in conc. H_2SO_4 with the same red-brown color as in lye but after a short time it became a dirty, dark brown-green. If the original red-brown solution were poured into H_2O, dark brown flakes precipitated, which left behind a

colorless solution after removal by filtration. The precipitate no longer behaved like *Cholepyrrhin*: it was easily soluble in alcohol, turning it green-brown, and it transmitted garnet red light. Added NH_3 and potash (K_2CO_3) did not change the color of the solution essentially, and the *Gmelin* color reaction failed. Only a pale red residue was seen at the layer bordering the HNO_3, which was yellow beneath, but yielded no green, blue, or violet coloration. Heated with a little soda lime (which is a mixture of 75% $Ca(OH)_2$, 20% H_2O, 3% NaOH, 1% KOH), *Cholepyrrhin* gave, besides NH_3, a tarry compound with a decidedly aniline-like odor; however, the presence of the latter could not be confirmed.

Maly reinvestigated *Städeler*'s *Biliprasin* (*135*), which, as may be recalled, appeared along with biliverdin during the various manipulations that he used to purify bilirubin from gallstones. *Städeler* found that biliverdin and *Biliprasin* were differentiated only by a color difference in alkaline solution, with the former being green and the latter being brown. However, *Maly* called into question the existence of *Biliprasin* since it was based only on an easily changeable and nuanced color, and because he never found a pure green alkaline solution of the biliverdin from isolated bile, rather, only when it was prepared from the purest *Cholepyrrhin*. Otherwise it was always brown-green. *Maly* then went on to describe three conditions under which *Cholepyrrhin* is converted to biliverdin: acids, alkalis, Br_2 and I_2. The last (halogens) represented what he believed to be novel reactions of *Cholepyrrhin*. The first two he explored again.

Some three years earlier, in 1865, *Maly* had reported that he was able to convert *Cholepyrrhin* completely to biliverdin by heating it in a mixture of $CHCl_3$ and acetic acid in a sealed tube in a water bath (presumably at 100°C). Since the reaction tube was only half full – the rest being air – he concluded that oxygen from the air was responsible, extolled the virtues of this simple transformation to very pure biliverdin, and concluded that biliverdin is an oxidation product of *Cholepyrrhin* – which others had concluded previously. He noted that other acids, such as HCl, will function in place of acetic acid to afford biliverdin in a less-clean transformation, and he speculated on whether the HCl in biliary vomitus might function likewise. Whether this "greening" due to an oxidation by means of oxygen was determined by or based on the influence of the acid he thought to contest by examining the influence of sulfurous acid (H_2SO_3) – because in the presence of this acid, a second compound (biliverdin) could in no way be formed by an oxidation. The experiment showed that biliverdin formation failed completely in the presence of H_2SO_3. Heating *Cholepyrrhin* in an aqueous or alcoholic solution of SO_2 in a water bath, either open to the atmosphere or in a sealed tube, gave no trace of "greening". (In the reaction open to the atmosphere, Professors *A.F. McDonagh* and *Jin-Shi Ma* produced yellow ranarubin, see Section 6 and references *168–170*). What dissolved in alcohol from these reactions with *Cholepyrrhin* was nothing other than golden yellow. *Maly* concluded from all the above that biliverdin formation still involved an oxidation process that requires oxygen in an amount sufficient for the relatively small amounts of *Cholepyrrhin* used.

More positive results came from using alkali. Thus, in an experiment reminiscent of that by *Tiedemann* and *Gmelin* (*48*), *Cholepyrrhin* in a dilute solution of

NaOH was divided into two parts, one was placed in a tube with air excluded by Hg, and the other was placed in a covered porous dish. After a few days, or even a month, the former still had a reddish brown color, while the latter had turned brownish green after a few days and precipitated green flakes of biliverdin upon addition of HCl. When a flask of oxygen was introduced into the glass bulb part of a glass cylinder, the gas was slowly but completely absorbed, leading exactly to a second and third "greening" of the solution. Other similar experiments were employed, *e.g*. using dilute soda lye (NaOH) in a U-tube, where only the end exposed to air turned green and the color change proceeded very slowly along the tube – an illustration of the diffusion of air through the liquid. These and other experiments convinced *Maly* that the question of oxygen uptake had been settled, and that the peculiar ability of oxygen from air to be absorbed and chemically bonded was nothing strange, because alkaline solutions of indigo white, gallic acid, and pyrogallic acid behaved just like *Cholepyrrhin* (in taking up oxygen) (*166*):

> Demnach betrachte ich die Frage von der Sauerstoffaufnahme als erledigt; die Eigenthümlichkeit den Luftsauerstoff zu absorbiren und chemisch zu binden hat, wie wir wissen, gar nichts seltsames; Indigweiss, Gallussäure und Pyrogallussäure in alkalischer Lösung verhalten sich eben so wie Cholepyrrhin. 129

The slow oxidation by atmospheric oxygen was compared to a more rapid oxidation by nascent oxygen. In an interesting experiment, an alkaline solution of (red-brown) *Cholepyrrhin* was stirred cautiously with PbO_2, and in two minutes the solution went over to green-brown; whereas, when the original solution was allowed to stand in air without PbO_2, the "greening" took place only after three to four or five days. At which point the addition of a little HCl and a lot of alcohol led to a biliverdin solution. Apparently the added PbO_2 considerably shortened the time-consuming reaction taking place in air alone. In the presence of stronger oxidizing agents, biliverdin itself suffered further oxidation. Thus, $KMnO_4$ gave further oxidation products. Though he did not realize it, *Maly* may have been the first to report a chemical degradation of the pigment to small fragment molecules (*166*, *167*): "Uebermangansaures Kali giebt sogleich weitergehende Oxydationsproducte" ($KMnO_4$ also gives further oxidation products).

Maly was thus able to obtain the green pigment that he called *Biliverdin* from *Cholepyrrhin* by: (i) heating in $CHCl_3$-glacial acetic acid solution in a sealed tube containing air; (ii) allowing an alkaline solution to stand in air a few days; and (iii) using PbO_2 as well as Br_2 as an oxidizing accelerant (*166*, *167*):

> Die *Darstellung* des Biliverdins kann nach dem Vorhergehenden verschiedene Wege einschlagen. 1) Entweder man erhitzt die chloroformige Cholepyrrhinlösung mit Eisessig in zugeschmolzenen Röhren, und wäscht, mit Wasser die Essigsäure weg; oder 2) man lässt die alkalischen Lösungen einige Tage an der Luft stehen, fällt mit Salzsäure und wäscht mit Wasser aus. Immer wurde zur weiteren Reinigung das Biliverdin in wenig starkem oder absolutem kalten Alkohol gelöst, von dabei etwa bleibenden braunen Flocken filtrirt, und mit Wasser vollständig ausgefällt. Der nun erhaltene flockige schwarzgrüne Niederschlag wurde noch mit Wasser, zuletzt mit Aether gewaschen.
>
> 3) Die oben erwähnte Einwirkung des Bleisuperoxyd's so wie die des Broms lassen sich noch zweckmässiger zur Darstellung des Biliverdins ausbeuten. Man rührt in die kalische Lösung des Cholepyrrhins langsam Bleisuperoxyd ein, bis eine Probe mit Säuren

eine rein grüne Fällung giebt, übersättigt dann das Ganze *schwach* mit Essigsäure, wobei unter vollständiger Entfärbung der Flüssigkeit Biliverdinblei niederfällt, das man abfiltrirt. Es wird dann gewaschen bis das Filtrat bleifrei ist, mit schwefelsäurenhaltigem Alkohol zerlegt, filtrirt und durch Wasser ausgefällt. 130

Maly described the characteristics of his pure biliverdin and provided its elemental combustion analysis (%C, H, N). As a powder it was dark green, odorless and tasteless, and somewhat hygroscopic. The purest biliverdin dissolved easily in alcohol (as well as in methanol), not with a brilliant green color but with more of a sap-green. But with a trace of added acid (HCl, H_2SO_4, glacial acetic acid) the color turned a beautiful clear green. Inorganic salts of calcium, lead, and silver could be prepared from the pigment in aq. ammonia. The pigment was soluble in alkali carbonates and hydroxides, giving a sap-green to brown-green color. When solid biliverdin was ground up with conc. H_2SO_4, the pigment dissolved to give a green color and was unchanged upon addition of H_2O, which precipitated flakes that produced a green color in alcohol. (One is led to believe that the biliverdin had undergone no chemical changes during the process.) It was soluble in ether to only an insignificant degree, insoluble in $CHCl_3$ but soluble in $CHCl_3$ containing a few drops of alcohol, soluble in glacial acetic acid-$CHCl_3$, and also in glacial acetic acid with an especially beautiful color. It was not soluble in benzene or CS_2, poorly soluble in amyl alcohol and in CH_3CH_2I – but easily soluble in the latter two if a little ethyl alcohol is added (*166*, *167*):

Das reine Biliverdin ist ein schwarzer glänzender, gepulvert schwarzgrüner Körper. Es ist geschmack- und geruchlos, und benetzt sich schwer mit Wasser. Bei 100º getrocknet giebt es etwas hygroskopische Feuchtigkeit ab, bleibt bei dieser Temperatur dann unverändert an Gewicht, ist aber so getrocknet sehr hygroskopisch.

Das reinste getrocknete Biliverdin löst sich in Alkohol nicht mit feurig grüner, sondern mit mehr saftgrüner Farbe. So wie aber dieser Lösung nur eine Spur einer Säure (Salz-, Schwefel-, Essigsäure) zugefügt wird, so wird sie prächtig rein grün.

Die alkoholische Biliverdinlösung giebt nach Zusatz von ein wenig Ammoniak mit Chlorcalcium einen dunkelgrünen in Wasser nicht löslichen Niederschlag; mit Silbernitrat eine flockige dunkelbraune Fällung unter vollständiger Entfärbung der Flüssigkeit. Dieses *Biliverdinsilber* löst sich nicht in Wasser, aber leicht in Ammoniak mit dunkelkastanienbrauner Farbe. Das auf ähnliche Weise mittelst Bleizucker dargestellte *Biliverdinblei* ist braungrün flockig.

Mit concentrirter Schwefelsäure verrieben löst sich das Biliverdin mit grüner Farbe, und wird von Wasser unverändert daraus in grünen in Alkohol löslichen Flocken ausgefällt.

In kohlensauren und ätzenden Alkalien löst es sich mit saftgrüner oder braungrüner Farbe. Es wird nur in unbedeutender Menge von Aether aufgenommen, und nicht von Chloroform, löst sich aber sehr leicht, sobald dem Chloroform nur einige Tropfen alkohol zugesetzt werden. Es löst sich ferner in Eisessig, in einem Gemenge desselben mit Chloroform und auch in gewöhnlicher starker Essigsäure, in diesen Flüssigkeiten mit besonders schöner Farbe.

Das Biliverdin ist nicht löslich in Benzol, Schwefelkohlenstoff, sehr wenig in Amylakohol und Jodäthyl, wohl aber leicht in beiden letzteren, wenn diesen ein wenig Aethylalkohol zugefügt wurde.

Methylalkohol löst das Biliverdin so leicht wie der gewöhnliche Alkohol. 131

The elemental combustion analysis showed the presence of 2% ash where only the %N is reported below (III and IV) but no ash in I and II. And using the atomic

mass convention where O = 16, *Maly* wrote that *Cholepyrrhin* added an oxygen atom to give biliverdin as $C_{16}H_{18}N_2O_4$. However, the actual %N of this formula is higher (9.26%) in N than that (8.74–8.77%) determined by experiment. Though this did not bother *Maly*, he took issue with *Städeler*, who had proposed the biliverdin formula $C_{16}H_{20}N_2O_5$, which would give a value of 60% for C (*166*, *167*):

Analyse.

I. 0,2400 Grm. Biliverdin gaben 0,561 Grm. Kohlensäure und 0,129 Grm. Wasser.
II. 0,2905 Grm. Substanz einer anderen Darstellung gaben 0,1585 Grm. Wasser.
III. 0,3356 Grm. Substanz einer dritten Darstellung gaben mit Natronkalk geglüht etc. 0,204 Grm. Platin.
IV. 0,3465 Grm. einer vierten Darstellung gaben eine 0,210 Grm. Platin hinterlassende Menge Platinsalmiak.

Diesen Resultaten entsprechen nach Abzug von circa 2 p.C. Asche bei III und IV (die Substanz von I und II war aschefrei) folgende Procentzahlen:

	I.	II.	III.	IV.
Kohlenstoff.....	63,74	–	–	–
Wasserstoff.....	5,97	6,05	–	–
Stickstoff.....	–	–	8,77	8,74

Würde das Cholepyrrhin wenn es in Biliverdin übergeht, ein Atom Sauerstoff (16. Gewth.) aufnehmen:

$$\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_3 + \text{O} = \text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_4,$$

so wäre die Formel des Biliverdins $\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_4$ und dieser entspricht die Berechnung:

Kohlenstoff.....	63,58
Wasserstoff.....	5,96
Stickstoff.....	9,26
Sauerstoff.....	21,19

welche mit den gefundenen Zahlen nur ein wenig im Stickstoffgehalt abweicht.

Nähme das Cholepyrrhin, wie Städeler angiebt, auch noch ein Molekül Wasser auf:

$$\text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_5 + \text{O} + \text{H}_2\text{O} = \text{C}_{16}\text{H}_{20}\text{N}_2\text{O}_5,$$

so würde der Kohlenstoffgehalt im Biliverdin bis auf 60,00 p.C. sinken. Ich glaube daher die erstere Formel für die richtige halten zu müssen. Die vollständige Erschöpfung meines Materiales, durch welche der Abschluss dieser ersten Abhandlung veranlasst ist, hindert mich vorläufig an einer letzten, noch nothwendigen Controlanalyse des Bilverdins. 132

Some six years later, when in Innsbruck, *Maly* read a paper before the Austrian Academy of Science in 1874, which appeared in a *Sitzungsbericht* (*171*) and was published a year later in *Annalen der Chemie und Pharmacie* (*172*) – a publication that summarized his careful studies on the conversion of bilirubin to biliverdin. These reports (*171*, *172*) were destined to be among his final three papers on bile pigments. Therein he described in great detail the isolation of bilirubin (by the year 1874, he had eschewed *Berzelius*' name for it, *Cholepyrrhin*) from ox gallstones. He noted that bilirubin was the major pigment, comprising some 28–48% by

weight after having been freed from its salts, mainly the calcium salt, and it appeared along with the typical other components, which he cited. The separation followed the methods of other workers, especially *Städeler* (*135*) and *Thudichum* (*152*). *Maly* added an improvement and was apparently the first to remove bilirubin by an extraction process using a continuous extraction apparatus (*171–173*) that operated on the same principle and design as the *Soxhlet* extractor.

Maly revisited the elemental combustion analyses of biliverdin that he reported in 1868 (*166*, *167*) and compared them to those published by *Thudichum* in the same year (*152*). *Städeler* (*135*) had reported no combustion analyses of biliverdin, but he nonetheless had proposed a formula ($C_{16}H_{20}N_2O_5$) for the pigment on the basis of *Heintz*'s earlier analysis in 1851 (*109*), and from this a %C, H, N, O could be calculated – for comparison purposes. *Maly*'s formula ($C_{16}H_{18}N_2O_4$) corresponded to a %N that was ~0.5% higher than the experimental value, and whereas the latter matched the value calculated from *Städeler*'s formula, *Thudichum*'s experimental %N matched that predicted by *Maly*'s formula. *Thudichum*'s formula ($C_8H_9NO_2$) for biliverdin, however, corresponded to one-half the *Maly* formula (*171*):

$C_{16}H_{18}N_2O_4$ = Bilirubin + O verlangt		Meine früheren Analysen gaben		Thudichum fand l.c.			Städeler's Formel $C_{16}H_{20}N_2O_5$ = Bilirubin + H_2O + O will	
		I	II	I	II	III		
C	63·58	63·74		63·08	62·09	62·14	C	60·00
H	5·96	5·97	6·05	6·25	6·12	6·00	H	6·25
N	9·26	N {8·77 u. 8·74		N {9·32 u. 9·36]			N	8·75
O	21·19						O	25·00.

133

In order to obtain a better combustion analysis of biliverdin, *Maly* prepared the latter from bilirubin in dilute aqueous Na_2CO_3 solution left in the presence of oxygen over a few days. He precipitated the green pigment by adding HCl and purifying (using its alcohol solubility) until it was ash-free upon combustion. Believing that his earlier nitrogen analysis fell short of the mark due to a failure of the method (incineration with copper oxide), he used the *Dumas* method and obtained a %N that nicely matched his formula. And on this basis, with the earlier %N deficiency having been explained and rectified, *Maly* then believed that the composition of his biliverdin should be considered as firmly established and definitive (*171*, *172*):

> Da sich bei meinen früheren Analysen eine Differenz nur im N gezeigt hat, der als NH_3 bestimmt worden war, mittlerweile aber von mehreren Seiten, so von Ritthausen und Kreusler . . . und namentlich von Nowak . . . constatirt wurde, dass gewisse Körper nur durch Glühen mit Kupferoxyd. ihren ganzen Stickstoff ausgeben, so wurde diesmal der N nach Dumas' Methode bestimmt.
>
> 1. 0·2785 Grm. Biliverdin, bei 100° getrocknet, gaben 0·6516 Grm. CO_2 und 0·1452 Grm. H_2O.
> 2. 0·3693 Grm. eines anderen Präparates gaben 31·5 CC. feuchten N bei 15° C. und 27·35 Par. Zoll.

	Gefunden		Berechnet $C_{16}H_{18}N_2O_4$
C.....	63·82		63.58
H.....	5·80		5·96
N.....		9·35	9·26.

Die geänderte N-Bestimmung hat also auch beim Biliverdin den kleinen Ausfall an N verschwinden machen, und da nun die Übereinstimmung in Bezug auf die verschiedenen Präparate, die Thudichum's und meinen Analysen zu Grunde liegen, eine ganz vollständige ist, so darf die Zusammensetzung dieses Körpers als definitiv festgesetzt betrachtet werden. 134

With this problem ostensibly behind him – although the apparent discrepancy between his formula and *Thudichum*'s (empirical) formula was unresolved and could not be resolved in the absence of knowing the molecular weight of the pigment – *Maly* turned to: (i) investigating a new method for converting bilirubin to biliverdin in the presence of oxygen and (ii) determining the material balance in the conversion. (i) Thus, heating pulverized bilirubin in molten $ClCH_2CO_2H$ (62°C) in the presence of air for a few days turned the melt green; addition of H_2O led to a green precipitate that was easily separated to leave behind an aqueous solution that contained only traces of pigment. In contrast, when the reaction was blanketed by CO_2, the color changed to brown, with no evidence of green. In two experiments, 0.7566 g bilirubin gave 0.7528 g biliverdin, and 0.4863 g bilirubin gave 0.4767 g biliverdin. The recoveries of pigment were 99.5% and 98.0%, which meant that very little was lost to the aqueous filtrate.

Then, yet another quantitative measure of the bilirubin to biliverdin conversion was determined – using bilirubin in dilute aq. Na_2CO_3. The green pigment, isolated by precipitation when HCl was added to the reaction, was dried, weighed, and compared to the weight of the dried bilirubin starting material. Traces of green pigment in the aqueous filtrate were estimated colorimetrically (*171*, *172*):

Bilirubin wurde in sehr verdünnter Sodalösung gelöst, unter gelegentlichem Einleiten von Sauerstoff einige Tage stehen gelassen, mit HCl das Biliverdin gefällt, am getrockneten und gewogenen Filter gesammelt und bis zum Verschwinden der Chlorreaction gewaschen. Es wurde dann bein 110° getrocknet und gewogen. Das grüngelbe Filtrat dampfte man ein und bestimmte darin den Gehalt an organischer Substanz durch schwaches Glühen des bei 125° getrockneten Rückstandes. Die Waschwasser, welche in dickerer Schichte auch eine Spur grüngelber Färbung zeigten, wurden colorimetrisch nach dem ersten Filtrate geschätzt. Dabei erhielt man:

Angewandtes Bilirubin (110° getrocknet).....	0·4558	Grm.	
Abfiltrirtes Biliverdin (110° getrocknet	0·4458	„	
Organische Substanz im Filtrate	0·0223	„	
Gesammtes Biliverdin	0·4681	Grm.	135

An estimated increase in weight of 2% – based entirely on the quantitative colorimetric analysis – was attempted to be correlated with the proposed stoichiometry for converting bilirubin to biliverdin: $C_{16}H_{18}N_2O_3 + O \rightarrow C_{16}H_{18}N_2O_4$, or an increase of 5.3%. From the current perspective, the quantitative determination of the

purported biliverdin left dissolved in the aqueous filtrate is clearly suspect and the experiment compromised. Nonetheless, *Maly* held to the belief that biliverdin contained one more oxygen than bilirubin (*174*):

> Jedenfalls stimmen also Analyse und Gewichtszunahme zusammen, und beide führen zu der Biliverdinformel $C_{16}H_{18}N_2O_4$, welche von der des Bilirubins durch einen Mehrgehalt von O sich unterscheidet. 136

In 1868, *Maly* had also explored the further oxidation of *Cholepyrrhin*, noting that (in his formula for biliverdin) only one atom of oxygen was added to yield the color change to green, *i.e.* the first stage of the *Gmelin* color change reaction. He thus contemplated that the subsequent color changes of the reaction were due to further oxidation, which to him meant the addition of more oxygen. Not an illogical extrapolation but one clearly based on the belief that: (i) the green color at the first stage of the *Gmelin* reaction was due to biliverdin, and (ii) the addition or incorporation of one atom of oxygen was responsible for the conversion of *Cholepyrrhin* to biliverdin. It was only later that (ii) was shown to be incorrect, that oxygen was in fact not incorporated.

In order to explore the colors of the *Gmelin* reaction, to attempt to stop the reaction at the various color stages, it was carried out using arsenic acid anhydride (As_2O_5) and HNO_2 to produce the usual color changes, which however concluded at the red coloration stage (and not the pale yellow), at a non-changing bright wine-red tone. Upon addition of H_2O at this stage, a bright, iron-oxide-colored flocculent precipitate ensued, but it could not be crystallized and thus remained of questionable purity. Nonetheless, it underwent an elemental combustion analysis which showed the new compound to be comparatively richer in oxygen than either *Cholepyrrhin* or biliverdin (*166*, *167*):

> . . . Ohne jetzt näher auf ihn einzugehen, will ich nur erwähnen, dass er in der That sehr viel sauerstoffreicher ist, als Cholepyrrhin oder Biliverdin, während Kohlenstoff und Wasserstoff zurücktreten. Folgende Zahlen zeigen dieses:

		Sauerstoff		Kohlenstoff	
Cholepyrrhin enthält	}	16,79	p.C.	67,13	p.C.
Biliverdin „	}	21,19	„	63,58	„
Neuer Körper „		30,39	„	55,23	„

> Mag nun dieser neue Körper nicht völlig rein erhalten worden sein, so viel zeigt seine Analyse sicher, dass die Oxydation noch weit über die Bildung des Biliverdins hinaus fortschreitet. 137

Difficulties were acknowledged in stopping the reaction at a given stage of color because the HNO_2 continued to effect oxidation. Later, *Maly* found a way to arrest all of the individual stages. This was accomplished using Br_2, which as described earlier, could oxidize *Cholepyrrhin* to biliverdin and beyond. Thus, addition of an alcohol solution of Br_2 led to a beautiful dark blue colored solution that remained unchanged for weeks. Addition of more of the alcoholic Br_2 produced a dirty violet color through clear dark red and finally a light wine-red. The series of color changes was much the same as that described previously from HNO_3 and from HNO_2. Although *Maly* indicated an ability to stop the color changes at individual

colored stages, he did not apparently isolate the corresponding pigments. Instead, he found that when the dark blue-colored $CHCl_3$ solution formed above was mixed with a $CHCl_3$ solution of *Cholepyrrhin* it simulated the clear green color of biliverdin but contained none of it. Evaporation in a dish separated blue and yellow rings, of which alcohol extracted only the blue, leaving behind the (yellow) *Cholepyrrhin*.

Maly concluded that there could be no doubt that the *Gmelin* reaction formed a series of compounds from *Cholepyrrhin* that contain increasingly more oxygen, from the single oxygen incorporated by biliverdin to blue and then red, and finally to the 30% O contained in the wine-red pigment. The violet he attributed to a mixture of red and blue. As described in his presentation to the Austrian Academy of Science in 1869, addition of Br_2 could be used to stop the oxidation at the blue stage (*174*). He believed he had found the means, using Br_2, to stop the progression and thus isolate pure pigments and said he would try to extend his research in that direction (*166*, *167*):

> Es kann sonach kein Zweifel sein, dass die bei der Gallenfarbprobe sich bildenden Körper weitere Oxyde des Cholepyrrhins darstellen, die zwischen Biliverdin und dem Körper der weinrothen Lösung mit 30 p.C. Sauerstoff stehend, mit diesen eine *mehrgliedrige an Sauerstoff zunehmende Reihe* bilden. Jedenfalls existiren noch ein blauer und rother Körper und das hellbraune Endproduct, während der violette wahrseheinlich ein Gemenge des rothen und blauen ist.
>
> Nachdem im Brom ein Mittel zu ihrer Fixirung und Reindarstellung gefunden ist, werde ich in dieser Richtung meine Versuche zu erweitern suchen. 138

Though *Maly* believed that he had achieved oxidation of *Cholepyrrhin* on the basis of a change in color, his thinking of oxidation was conditioned by processes involving the incorporation of oxygen. *Städeler*, too, and his predecessors were similarly inclined, as was *Thudichum*. But by the time that *Maly* began his work on bilirubin, *Städeler* was absenting himself from bile pigment research. Not so with *Thudichum*, who began to follow *Maly*'s published work, and as will be seen, became exasperated by it and the multitude of errors he believed to have found in it. Eventually, neither *Maly* nor *Thudichum* were proved correct in the concept of oxidation as applied to bile pigments.

Maly also had an interest in reduction, though he was not alone in this. His reduction of biliverdin appears to be novel for its period in time.

In 1868, he explored the reduction of biliverdin using spongy platinum, apparently freshly precipitated and activated. Thus, treatment of the pigment with the Pt over a period of a few days to a few hours gave a red-brown solution, seen after screening out the spongy Pt. (Whether the product was bilirubin or one of further reduction was not further stated.) (*166*, *167*):

> Platinschwamm reducirt die Biliverdinbildung von einigen Tagen auf einige Stunden; hat man die rothbraune Lösung in einer flachen Schale, so siebt man vom hineingeworfenen Platinschwamm aus die Farbenumwandlungen vor sich gehen. 139

Bilirubin, too, was shown by *Maly* to suffer reduction, albeit much less readily than biliverdin. Thus in his studies from Innsbruck, where he was *Professor der Physiologischen Chemie* at the university from 1869 to 1875, he published his preliminary work on the synthetic transformation of bilirubin into the pigment of urine

in an article submitted on February 26, 1872 (*175*). He then followed it with a longer article, also submitted in February 1872 to the same journal (*176*). Thus, as *Maly* wrote while transitioning the pigment's names, *Cholepyrrhin* (bilirubin that had been isolated from ox gallstones and purified, as described earlier) was dissolved, or in later experiments suspended, in dilute aq. KOH or NaOH, protected from air, and allowed to react with the nascent hydrogen evolved upon addition of Na(Hg). (The author carried out essentially the same reaction of bilirubin in the *C.J. Watson* lab at the University of Minnesota Medical School in 1964–1965.) At first the procedure revealed no H_2 evolution (because it was being taken up by bilirubin). Later, as the reaction progressed, and the original opaque, dark solution had cleared and become a light brown color, it could be shown that the reaction vessel contained evolved hydrogen. After 2–4 days, during which excess Na(Hg) had been added, with frequent shaking at room temperature, and with subsequent gentle warming until no further lightening of the color could be observed, the Hg was removed and excess HCl (or acetic acid) was added. This produced a garnet red color, showing that bilirubin had undergone a change, and dark red-brown flakes separated, leaving a red-colored solution. The precipitate was filtered and washed to remove NaCl entirely. The collected precipitate had the characteristics of a weak acid, as it dissolved easily in ammonia or alkali with a yellow-brown color. Unlike bilirubin, however, it was readily soluble in alcohol with a reddish color, in $CHCl_3$ with a yellow-red color, and in alkaline solution with a brown color. This new pigment was submitted to combustion analysis from which a formula ($C_{32}H_{40}N_4O_7$) was calculated from the assumption that two bilirubin molecules had together absorbed one molecule each of H_2 and H_2O. *Maly* named the new pigment *Hydrobilirubin* (hydrobilirubin) (*176*):

> Zur *Analyse* wurden Proben von Substanz genommen, die von dreierlei Darstellungen herrührten, und bei welchen das Auflösen in Alkali und Ausfällen mit Säuren bald einmal, bald zwei- und dreimal vorgenommen war.
>
> 1. 0,2193 Grm. Substanz gaben 0,523 CO_2 und 0,1404 H_2O.
> 2. 0,2652 Grm. Substanz gaben 0,1646 H_2O.
> 3. 0,2262 Grm. Substanz gaben 0,1474 Platin.
> 4. 0,2483 Grm. Substanz gaben 0,5886 CO_2 und 0,1559 H_2O.
> 5. 0,2174 Grm. Substanz gaben 0,5142 CO_2 und 0,1347 H_2O.
>
> Auf Procente bezogen:

	1.	2.	3.	4.	5.	Mittel
C	64,89	–	–	64,65	64,50	64,68
H	7,09	6,80	–	6,98	6,87	6,93
N	–	–	9,22	–	–	9,22.

> Diese Zahlen stimmen so gut untereinander überein, dass die Reaction als eine sehr glatte bezeichnet werden muss. Die Substanz is kohlenstoffärmer und wasserstoffreicher als Bilirubin, entsprechend ihrer Bildung und kann also nur durch Bindung von Wasserstoff entstanden sein. Nimmt man, an dass auch noch Wasser eingetreten ist, und zwar H_2O auf 2 (Mol. ?) Bilirubin neben H_2, so würde der Körper $C_{32}H_{40}N_4O_7$ resultiren, nach der Gleichung:

$$2\ C_{16}H_{18}N_2O_3 + H_2 + H_2O = C_{32}H_{40}N_4O_7$$

und dieser verlangt:

		Gefunden (Mittel)
C_{32}	64,86	64,68
H_{40}	6,75	6,93
N_4	9,45	9,22
O_7	–	–

was mit den erhaltenen Resultaten recht gut übereinstimmt. Der neue Körper ist also durch Aufnahme von Wasserstoff und Wasser unter Verdoppelung des Moleculs von Bilirubin (falls nicht, wie vielleicht wahrscheinlicher, die Bilirubinformel doppelt so gross als gewöhnlich zu schreiben ist) entstanden und soll fortan als *Hydrobilirubin* bezeichnet werden. 140

Various other properties of hydrobilirubin were investigated, including those customary for the times: salts with a wide range of various metal ions, from alkali to heavy metals. One property not shared with its precursor, bilirubin, was the strong fluorescence exhibited by certain of the salts, especially those formed from $ZnCl_2$ or $ZnSO_4$ in aq. NH_3. The pigment did not exhibit the *Gmelin* reaction, but elementary colorimetry was investigated (and will be described later). Of considerable importance to *Maly* was a probable relationship between his hydrobilirubin and the pigment of urine, *i.e.* what *Jaffe* termed *Urobilin* (urobilin). In comparing the various characteristics of hydrobilirubin and urobilin, *Maly* found them to be identical, though he preferred the former name (his) to the latter because it expressed something more of its constitution (*176*):

Durch die Wiederholung dieser Stellen aus Jaffe's Abhandlung habe ich am Besten gezeigt, dass die Eigenschaften meines Hydrobilirubins und Jaffe's Urobilins, also die Substanzen selbst identisch sind. Dass ich für meine Substanz (richtiger für beide) den Namen Hydrobilirubin einführe, begründet sich durch die künstliche und natürliche Bildung, und zweitens dadurch, dass wenigstens etwas von der Constitution durch den Namen ausgedrückt ist. 141

Maly also noted that *Thudichum* had isolated a compound from urine that he named *Urochrom* (urochrome) but had little discussed it, aside from noting its yellow-red color. More important to him, however, was *Scherer*'s work (*93*) on the urinary pigment. Apparently repeating *Scherer*'s isolation procedure for fresh urine from feverish patients, *Maly* found *Scherer*'s pigment and hydrobilirubin to have identical characteristic properties and rather similar %C and H in combustion analysis: %C 65.25; %H 6.59; and %C 64.99; %H, 7.00 for *Scherer's* urinary pigment, or not far removed from the %C 64.68; %H 6.93 for hydrobilirubin. Yet other analyses gave results in poorer agreement (*176*):

. . . was nicht weit entfernt von der Zusammensetzung des Hydrobilirubins ist, und darauf deuten würde, dass das Scherer'sche Präparat wenigstens keine grossen Mengen verunreinigender Substanz enthielt. Anderer Analysen freilich gaben weiter abstehende Resultate *).

*) Damit ist auch wenigstens für den wichtigsten Harnfarbstoff der angebliche Eisengehalt widerlegt. Auch hat Dr. Schlemmer neuerdings wieder in meinem Laboratorium in grösseren Mengen Harns vergeblich nach Eisen gesucht. 142

It may be interesting to note that *Maly* assumed, given the identity of hydrobilirubin with the urinary pigment, that the circulation of bilirubin took it to the gut, where it was reduced (hydrogenated) by the hydrogen produced there and added H_2O to form the hydrobilirubin that later appeared in urine. (He also found that treatment of biliverdin with Na(Hg) led to entirely similar results as with bilirubin: both formed a brown solution.) *Maly* thus explained that the hydrobilirubin was absorbed from the gut and went finally into the urine, thereby to end its cycle in the organism. He assumed that hydrobilirubin formed in the gut played no important role there and was only a means to bring the compound to excretion from the organism; *i.e*. the hydrobilirubin was absorbed from the gut, where it apparently played no role, and finally went into the urine. Bile pigments could thus be viewed as useless by-products of liver metabolism (*176*):

> Indem wir so gesehen haben, in welch naher chemischer Beziehung der Orange-Gallenfarbstoff und der (hauptsächlichste) Harnfarbstoff wenigstens beim Menschen zu einander stehen, ergiebt sich der Kreislauf dieser Pigmente von selbst, und manche zusammenhanglose Thatsache reiht sich schön ein. Das mit der Galle in den Darm ergossene Bilirubin erleidet während seiner Wanderung herab bis zum Colon und in diesem selbst seine Wasserstoff- und Wasseraufnahme unter dem Einflusse von Wasserstoff entbindenden Processen. Ganz gleich verhält sich Biliverdin: ich habe eine alkoholische Biliverdinlösung mit Natriumamalgam behandelt, und bald eine braune Lösung erhalt, identisch mit der aus Bilirubin. . . .
>
> Vom Darm aus wird das Hydrobilirubin aufgesaugt und geht schliesslich in den Harn, um dort seinen Cyclus im Organismus zu beenden. Da das Hydrobilirubin im Darm keine ersichtliche Rolle spielt, und die Aufsaugung nur ein Mittel ist den Körper aus dem Organismus hinaus zu bringen, so ist nicht einzusehen, dass die Gallenfarbstoffe überhaupt einem Zwecke dienlich sein sollten, und man wird dermalen sie nicht anders denn als nutzlose Nebenproducte des Leberchemismus anzusehen haben. 143

Maly's experiments involving bilirubin and biliverdin, especially the oxidation reactions that produced the latter from the former, and their formulas derived from the elemental combustion analyses, drew sharp criticism from *Thudichum*. So did hydrobilirubin. But what piqued *Thudichum*'s interest and ire most were *Maly*'s reactions of bilirubin with Br_2. In his work published in 1868 (*166*, *167*), *Maly* mentioned a third route for converting *Cholepyrrhin* to biliverdin, a route destined to provoke controversy and a polemic from *Thudichum*: oxidation using Br_2 or I_2. To accomplish such a transformation, described by *Maly* as surprisingly nice, *Cholepyrrhin* was allowed to stand in Br_2 vapor mixed with moist air. This resulted in rapid darkening and yielded a compound no longer soluble in $CHCl_3$ but one that dissolved in alcohol with a clear green color. (No mention was made as to whether the *Cholepyrrhin* used was as a solid or in solution.) The reaction described was allowed to continue, but *Maly* found it advantageous to carry out the transformation of a yellow solution of *Cholepyrrhin* in $CHCl_3$ using a decently dilute solution of Br_2 in alcohol. Dropwise addition of the latter into the former caused immediate darkening of the $CHCl_3$ solution to a sap-green color. Careful addition led to a point where the $CHCl_3$ solution was a clear, beautiful bright green, with the biliverdin formed remaining in the $CHCl_3$-alcohol mixture. At this point *Maly* claimed that all

of the *Cholepyrrhin* had been converted to biliverdin as the solution was stable for weeks (*166*, *167*):

> Ich habe erwähnt, dass es ausser Säuren und Basen noch eine dritte Reihe von Körpern giebt, welche Biliverdin aus Cholepyrrhin erzeugen; es sind diess die Haloide Brom und Jod. Namentlich überraschend schön ist die Umwandlung mittelst Brom. Bringt man Cholepyrrhin unter eine Glasglocke, in der sich mit feuchter Luft gemischter Bromdampf befindet, so färbt es sich bald dunkel, und wird nicht mehr von Chloroform, aber von Weingeist mit rein grüner Farbe gelöst. Da aber dabei die Bromwirkung leicht etwas zu weit geht, so kann man den Versuch viel vortheilhafter in folgender Weise anstellen. Man versetzt eine gelbe chloroformige Cholepyrrhinlösung mit einer recht verdünnten alkoholischen Lösung von Brom. Schon die ersten Tropfen machen die Flüssigkeit dunkel saftgrün, und es lässt sich sehr leicht bei weiterem vorsichtigen Bromzusatz der Punkt treffen, bei dem die ganze Flüssigkeit ein reines prachtvoll feuriges Grün zeigt *). In diesem Momente ist alles Cholepyrrhin in Biliverdin übergegangen, und die Flüssigkeit kann wochenlang stehen, ohne sich zu verändern. 144
>
> *) In diesem Gemenge von Chloroform mit nur wenig Alkohol bleibt das Biliverdin gelöst.

Thus, reaction of bilirubin with Br_2 was viewed by *Maly* to be an oxidation because it produced a green pigment thought to be biliverdin, a known oxidation product of bilirubin, for he knew that halogens in the presence of moisture cause oxidation of oxidizable compounds. And what atmospheric oxygen brought about so slowly, the conversion with Br_2 took a few seconds.

Some 15 months after his July 9, 1874 presentation to the Austrian Academy, *Maly*, then *Professor der allgemeinen Chemie* at the TH-Graz, made his final presentation on bile pigments on October 17, 1875 (*177*), which was published in 1876 in the *Annalen der Chemie und Pharmacie* (*178*). The subject was the treatment of bilirubin with halogens, especially Br_2, and it drew a lambasting from *Thudichum*, as will be noted later. Although *Maly* had first believed that bilirubin was oxidized to biliverdin by reacting with Br_2, from which a blue coloration gradually became evident, by reinvestigating this erstwhile "oxidation" reaction, he became convinced that the green color of reaction was actually due to a mixture of a blue compound and unreacted bilirubin. Thus, with careful control of the ratio of added Br_2 as a solution in $CHCl_3$ to a solution of bilirubin in $CHCl_3$, with an added few drops of alcohol, he conducted a series of reactions from which each step in the sequence of *Gmelin* color changes was observed (*178*): "Es zeigten sich brillante farbige Lösungen von grosser Haltbarkeit und in der Reihenfolge, wie sie bei der Gmelin'schen Salpetersäurereaction auftreten" (It exhibits brilliantly colored solutions of great stability and in the sequence that appears in the *Gmelin* HNO_3 reaction). From a stable "blue step" produced by reaction with an appropriate amount of Br_2, the solution was observed to run through the remainder of the color steps of the *Gmelin* reaction, from red to yellow-brown. Based on his bromination experiments, *Maly* concluded that the blue pigment did not arise by oxidation but by bromination – and that it was a very bromine-rich new compound (*178*): "So begreift sich, dass es für den sich damit Beschäftigen denn viel Ueberraschendes hatte, zu finden, dass die Bromwirkung dabei keine oxydirende

ist und der blaue dabei entstehende Körper eine an Brom sehr reiche Verbindung ist" (So it is understandable that it was very surprising to find that the action of the bromine used is not oxidizing and the blue compound arising is a substance very rich in bromine).

Maly set about to prepare, isolate, and characterize the blue pigment from bromination of bilirubin and achieved (i) good success with a procedure that involved addition of a few drops of Br_2 to bilirubin suspended in ether, and (ii) even better success with bilirubin suspended in alcohol-free $CHCl_3$ and addition of Br_2 in the same solvent. The elemental combustion analysis revealed that although the %C varied considerably (35.51–47.83% among the six C,H analyses performed), the %H (4.14–4.7%) did not; moderate consistency was found among the seven Br analyses (27.70–29.60%); and the two N analyses gave 7.4% and 7.8%. From those data, *Maly* concluded that the blue compound was a tribromo derivative of bilirubin, wherein three hydrogens had been lost and replaced with three bromine atoms to give a formula $C_{32}H_{33}Br_3N_4O_6$ (*178*):

$$2\ C_{16}H_{18}N_2O_3 = C_{32}H_{36}N_4O_6$$

$$C_{32}H_{36}N_4O_6 + 3\ Br_2 = C_{32}H_{33}Br_3N_4O_6 + 3\ HBr$$

C_{32}	47,46
H_{33}	4,08
Br_3	29,66
N_4	6,92
O_6	11,88.

In order to reach the formula, *Maly* had to assume a doubling of his bilirubin "basic" formula ($C_{16}H_{18}N_2O_3$) to $C_{32}H_{36}N_4O_6$. This, and the hydrobilirubin formula ($C_{32}H_{40}N_4O_7$) derived from twice the original formula plus H_2 + H_2O, induced him to rethink his formula for bilirubin. He concluded that the original "basic" formula could not be maintained and settled on $C_{32}H_{36}N_4O_6$ as the most appropriate (*178*):

> Bei solcher Zusammenstimmung bin ich wohl berechtigt zu behaupten, dass die bisher übliche Formel des Bilirubins, welche durch $C_{16}H_{18}N_2O_3$ ausgedrückt wurde, nicht aufrecht gehalten werden kann, sondern dass dieselbe verdoppelt werden müsse. Dieselbe wird dann $C_{32}H_{36}N_4O_6$, und man hat:
>
> Bilirubin $C_{32}H_{36}N_4O_6$,
> Tribrombilirubin $C_{32}H_{33}Br_3N_4O_6$
> Hydrobilirubin $C_{32}H_{40}N_4O_7$. 145

Maly's formula is about as close to the correct formula ($C_{33}H_{36}N_4O_6$) for bilirubin as anyone had reached by 1875. But there were others, including *Thudicum*, who held to a formula different from *Maly*'s. Yet before he bowed out of bile

pigment research, *Maly* initiated some of the earliest spectroscopic investigations of bilirubin and its derivatives.

2.10 The Emergence of Bile Pigment Spectroscopy: Colorimetry and Its Applications

Städeler was the last of the well-known investigators of bilirubin to employ no spectroscopic measurements, though he was well acquainted with and used the other available analytical technique: elemental combustion analysis. *Maly* and *Thudichum*, whose investigations of bilirubin followed very closely to those of *Städeler*, were apparently the first to employ the new analytical technique, *spectrum analysis*, in their studies. Spectrum analysis, the precursor to ultraviolet-visible spectroscopy, was based on absorption of light in the visible region of the electromagnetic spectrum – specifically the colors seen when sunlight is dispersed through a 60° prism. When a solution of a colored substance is positioned in the light beam and before the prism, certain of these colors are reduced in intensity or extinguished by the substance absorbing the complementary color of light. Hence absorption colorimetry.

Absorption colorimetric measurements of the day made use of early instrumentation due to *Bunsen*,[49] *Kirchhoff*,[50] and *von Steinheil*[51] that followed a report on October 20, 1859 to the Royal Prussian Academy of Sciences (*Königliche Preussische Akademie der Wissenschaften*) by *Bunsen* and *Kirchhoff*. *Bunsen*, who with his laboratory assistant *Peter Desaga*, designed the *Bunsen* burner which gave a hot, clean flame, and *Kirchhoff*, who studied thermal radiation and coined the phrase "black body radiation," jointly studied the emission spectrum of heated elements and laid the basis for the emergent field of "spectrum analysis" to become used as a new analytical technique in biological chemistry for characterizing substances, together with elemental combustion analysis. A month later, on 19 November 1859, *von Steinheil* was asked by *Bunsen* and *Kirchhoff* to fabricate an

[49] *Robert Wilhelm Eberhard Bunsen* was born on March 30, 1811 in Göttingen and died on August 16, 1899 in Heidelberg. In 1836 he succeeded *Friedrich Wöhler* at Kassel and in 1852 he succeeded *Leopold Gmelin* at the University of Heidelberg.

[50] *Gustav Robert Kirchhoff* was born on March 12, 1824 in Königsberg and died on October 17, 1877. He was a physicist and professor at Breslau. In 1854 he was called to the University of Heidelberg where he collaborated with *Bunsen*, and in 1875 he accepted the first chair in theoretical physics at Berlin.

[51] *Carl August von Steinheil* (1801–1870), was a physicist, Professor of Mathematics in Munich from 1832, and scientific instrument builder.

instrument to examine the "fixed lines" of the solar spectrum. The primitive spectroscope using a prism to disperse the incident light (*179*) was thus built to serve the scientific investigations of *Bunsen* and *Kirchhoff*. It allowed *Hoppe-Seyler*[52] at the University of Tübingen to study the absorption of solutions of colored substances held in a rectangular cuvette and positioned between the (sunlight) light source and collimating telescope (*180*). The apparatus used, which arose from studies of the visible part of the electromagnetic spectrum, was thus limited mainly to colored substances – of which the yellow, green, and other colors of the bile pigments were ideal candidates for analysis.

The spectrum analysis scale for the visible region was adjusted to the *Fraunhofer*[53] emission lines from certain elements, *e.g.* Kα (7685 Å), Liα (6705 Å), Na (5892 Å), Sr (4607 Å), Ca (4226 Å), *etc*. Thus, the *Bunsen-Kirchhoff* scale, which ranged from 17.5 to 166.0, could be calibrated, *e.g.* the sodium D-line above corresponded to 50 on the *Bunsen* scale. The colors of the scale ranged of course from one extreme end of the spectrum, the red, identified by the *Fraunhofer* line "A" and corresponding to the potassium Kα line (seen in a flame test), or to 17.5 on the *Bunsen-Kirchhoff* scale, and ended at the other, the violet, identified as *Fraunhofer* line "H_2" at its extreme, or 166.0 on the *Bunsen-Kirchhoff* scale (*181*):

> In sunlight, which is thrown horizontally upon the slit by a heliostat, Fraunhofer's lines may be employed, the most characteristic of which are shown in the

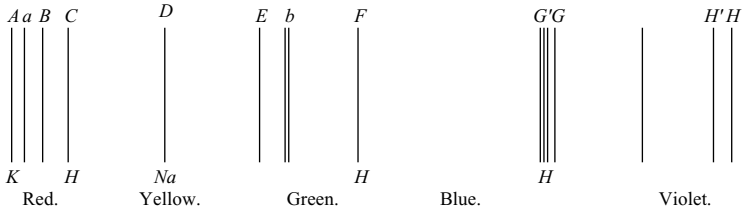


Fig. 8.

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[52] *Ernst Felix Immanuel Hoppe-Seyler* was born on December 26, 1825 in Freyburg an der Unstrut, Saxony and died on August 10, 1895 in Wasserburg am Bodensee, Bavaria. He was perhaps the pre-eminent physiological chemist of the 19th century. Trained as a physician, he received the Dr. med. in 1850 in Berlin after studies at the Universities in Halle, Leipzig, Berlin, Prague, and Vienna. He practiced medicine, habilitated at Greifswald in 1855, and in 1856 was *Assistent* to *Rudolf Virchow* at the Pathological Institute in Berlin, then in 1857 director of the chemical laboratories at *Virchow*'s newly established Pathological Institute of the Berlin Charité, where he was appointed a. o. Professor in 1860. He was appointed a. o. Professor of Applied Chemistry at Tübingen in 1861, then o. Professor until he accepted a call in 1872 as o. Professor of Physiological Chemistry at the newly-established University of Strassburg, where he remained until his death due to a stroke at his house in Wasserburg. In 1877, he founded the respected journal *Zeitschrift für Physiologische Chemie*, that became know after his death as *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*. Born *Ernst Hoppe*, his mother died when he was a child, and he added *Seyler* to his name after he was adopted by his brother-in-law.

[53] *Joseph von Fraunhofer* (1787–1826), the Bavarian optician, invented the spectroscope and discovered 574 dark lines (above absorption) appearing in the solar spectrum that are still called *Fraunhofer* lines.

accompanying figure in their relative positions, as seen through a flint-glass prism. In order to see *A* and *a* the slit must not be too narrow, and a red glass should be held before it. With a narrow slit and greater magnifying power, *D* is seen to be a very close double line.

Where sunlight cannot be used, the line *A* may be obtained by means of the potassium flame, *D* by the sodium flame, *C*, *F*, and *G′* by the light of the electric spark in a narrow Geissler's tube filled with rarefied hydrogen.

These thus correspond to the red and near ultraviolet ends of the visible spectrum, where the wavelengths correlate approximately to the *Bunsen-Kirchhoff* scale as explained long ago by *Kohlrausch* (*181*):

TABLE 19.

LINES OF THE FLAME-SPECTRA OF THE MOST IMPORTANT LIGHT METALS,

according to Bunsen and Kirchhoff's scale; the sodium-line being taken as 50, and the slit having a breadth of 1 division.

The first number denotes the position of the middle of the line upon the scale, the Roman figure indicates the brightness, I being the brightest, and the third number gives the breadth of the band when it exceeds 1 scale-division, the breadth of the slit.

S signifies that the line is quite sharp and clearly defined, *s* that it is tolerably so; the remaining lines being nebulous and ill defined.

The lines most characteristic of each body are printed in thick type.

The brightness of the lines of *Ca*, *Sr*, and *Ba* is that of a constant spectrum. If the chlorides be employed, the spectra are at first much brighter. In many cases the flame-spectra are really those of compounds, the spectra of the metals themselves obtained by the electric spark being frequently entirely different, and consisting of much finer lines.

The colours of the spectrum are approximately—red to 48, yellow to 52, green to 80, blue to 120, and violet beyond.

K.	*Na.*	*Li.*	*Ca.*	*Sr.*	*Ba.*
17·5 II. *s*		**32·0 I.** *S*	33·1 IV. 2	29·8 III.	
			36·7 III.	32·1 II.	
				33·8 II.	
Faint continuous spectrum	**50·0 I.** *S*	45·2 IV. *s*	**41·7 I. 1·5**	36·3 II.	35·2 IV. 2
from 55 to120			46·8 III. 2	38·6 III.	41·5 III. 3
				41·5 III.	45·6 III.*s* 1·5
			49·0 III.	**45·8 I.**	
					52·1 IV.
			52·8 IV.		56·0 III. 2
			54·9 IV.		**60·8 II s**
			60·8 I. 1·5		66·5 III. 3
			68·0 IV. 2		**71·4 III. 3**
				105·0 III. *s*	**76·8 III. 2**
153·0 IV.			**135·0 IV.** *S*		82·7 IV. 4
					89·3 III. 2

TABLE 19*a*.
WAVE-LENGTHS OF THE PRINCIPAL LINES OF THE SOLAR SPECTRUM IN TENTH-METRES IN AIR AT 760 MM. PRESSURE AND 16° TEMPERATURE (Angström)

In order to obtain the wave-lengths in vacuo the numbers must be multiplied by the respective refractive indices of the rays for air at 16° C. (Watts).

			Approximate Positions on Bunsen and Kirchhoff's Scale.
A	7604	1^{-10} metre	17·5
B	6867	„	27·6
C	6562	„	34·0
D_1	5895	„	50·0
D_2	5889	„	
E	5269	„	71·0
b_1	5183	„	75·7
F	4861	„	90·0
G	4307	„	127·5
H_1	3968	„	162·0
H_2	3933	„	166·0

TABLE 19*b*.
Wave-Lengths of some of the Principal Bright Lines in the Spectra of the Elements, and their Approximate Positions on Bunsen and Kirchhoff's Scale.

Element.	Wave-Length.		Scale Number.	
Kα	7685	1^{-10} metre	17·5	
Liα	6705	„	32·0	
Hα	6562	„	24·0	
Liβ	6102	„	45·2	
Na	5892	„	50·0	
C	5662	„	58·	Edge of band seen in blue of candle flame.
Tl	5348	„	67·	
C	5170	„	75·	Edge of band in candle flame.
Hβ	4861	„	90·	
Sr	4607	„	105·	
Ca	4226	„	135·	Approximate in flame spectrum.
Hγ	4101	„	151·	
Kβ	4080	„	153·	Flame spectrum.

With *Beer*'s earlier report on the transmission of light through colored solutions (*182*), the stage had been set for the evolution of spectrum analysis to a quantitative level, as promoted by *Bunsen*. *Bunsen* found it possible to gain quantitative information with use of a standard reference and sample dilutions, which led him to the notion of a molar absorptivity extinction coefficient (*183*). Subsequently, almost

inevitably as spectral analysis became widely used, the instrumentation evolved in stages to more modern types by *Vierodt*, *d'Arsonval*, *Duboscq*, *Zeiss*, *etc*. (*184*).

In 1868, in his first long paper (*166*, *167*), *Maly* reported on what might be the earliest (visible) absorption spectra of *Cholepyrrhin* (bilirubin) and biliverdin. He indicated that a $CHCl_3$ solution of the former extinguished (light absorbed by the sample solution, not transmitted through it) the entire blue and violet regions up to approximately line 70 on the *Bunsen* scale (or from ~3900 to 5300 Å); whereas, more dilute solutions removed only the violet. A similar behavior was seen in aq. NH_3. Though it was possible in the 1860s to prepare accurately weighed solutions, there was no indication of measured concentrations of the solutions, possibly because there was no concept of an exact quantitative relationship between sample concentration, the incident and exit light intensities, and the thickness (pathlength) of the sample solution (*Beer-Lambert* law). In any event, at best only a qualitative or only vague quantitative reference was typically expressed in terms of regions of the visible spectrum having been extinguished, which meant that all of the available light had been absorbed. At times a reference standard was invoked for comparative purposes. The solutions of *Cholepyrrhin* were said to have a color approximating a concentrated solution of acidic K_2CrO_7, and at the corresponding concentration the field of vision of the spectroscope was completely extinguished from the violet end to the sodium D-line (50 on the *Bunsen* scale, or ~ 5889 Å) and fairly sharply defined. If the solution were dilute it generally appeared yellow or green, but somewhat blurred. When the solutions that were very dilute such that the "coloring power" of *Cholepyrrhin* in NH_3 solution contained barely measurable traces, the lamp light appeared nearly colorless, but a good part of the violet was still extinguished (*166*, *167*):

Absorptionsspectra der Gallenfarbstoffe.

Eine Chloroformige Cholepyrrhinlösung vor den Spalt eines Spectralapparates gebracht, löscht das ganze Blau und Violett aus, bis etwa zur Linie 70 nach der Bunsen'schen Scala. Sehr verdünnte eben noch gelbe Lösungen nehmen noch das Violett hinweg.

Lösungen von Cholepyrrhin in wässerigem Ammoniak verhalten sich ähnlich. Sind sie so gefärbt wie etwa eine concentrirte Lösung von saurem chromosauren Kalium, so erscheint das Sehfeld von violetten Ende bis nahe an die Natriumlinie (50) vollständig schwarz, und ziemlich scharf abgegrenzt; wird die Lösung verdünnt, so erscheint allmählich gelb und grün, aber etwas verwischt. Selbst Lösungen, die so verdünnt sind, dass sie bei Lampenlicht fast farblos erscheinen, also bei der färbenden Kraft des Cholepyrrhins in ammoniakalischen Lösungen . . . kaum mehr wägbare Spuren enthalten, löschen noch einen guten Theil von Violett aus. 146

From these data it seems clear that *Maly*'s reddish solutions of *Cholepyrrhin* were very concentrated, and they blanked out or extinguished the blue-violet region of the spectrum. *Maly* also performed a spectrum analysis of biliverdin. Thus an alcoholic solution of biliverdin was found to exhibit absorption at both ends of the spectrum. Through strongly colored layers or films, only green light was transmitted. In somewhat more dilute solutions, first yellow, orange, and a part of the red, later blue and violet. The outermost red was still removed by very dilute solutions, but no specific references to the *Bunsen-Kirchhoff* scale were cited (*166*, *167*):

Biliverdin in alkoholischer Lösung zeigt Absorptionen nach beiden Enden des Spectrums. In stark gefärbten Schichten geht nur grünes Licht hindurch, in etwas verdünnteren erscheint

zunächst gelb, orange und ein Theil des Roth, später blau und violett; das alleräusserste Roth wird noch von sehr verdünnten Lösungen hinweg genommen. 147

All this was from 1868, within a decade of *von Steinheil's* building a spectroscope and not long after *Hoppe-Seyler*'s report on his spectral studies of blood and hematin (*180*). Shortly after his report on the spectrum analysis of *Cholepyrrhin* and biliverdin, *Maly* applied the emerging spectroscopy to hydrobilirubin, as reported in 1872, for purposes of comparing it to *Jaffe*'s urinary pigment, urobilin (*185*). The former, dissolved in alcohol or dilute aqueous ammonia or sodium phosphate to give a yellow, or a red-yellow or rose color, was placed in the spectroscope in 0.5–2.0 cm (pathlength) cuvettes. The solution showed a vivid and marked spectral absorption between green and blue, between the *Fraunhofer* lines b and F, or between 5183 and 4861 Å (*175*):

Löst man etwas Hydrobilirubin in verdünnten Alkohol, oder setzt man zu einer so verdünnten alkalischen Lösung (in Ammoniak oder phosphorsaurem Natron u. s. w.) deselben, dass Säuren nichts mehr ausfällen, etwas Salz- oder Essigsäure bis zur sauren Reaction, d. h. so weit, dass die Flüssigkeit die gelbe Farbe verliert und rothgelb oder rosenfarbig wird, so zeigt sie in dünner Schicht (½ bis 2 CM.) vor den Spectralspalt gestellt eine sehr lebhafte und markirte Absorption des Spectrums zwischen grün und blau, und zwar bei meinem grössen Apparat (wenn Li bei 102,5; Na auf 120 und K-β auf 219,5 steht) innerhalb der Theilstriche 146 bis 160, oder allgemeiner ausgedrückt genau zwischen den Fraunhofer'schen Linien b und F. Eben so bleibt es wenn die Lösung stärker sauer wird; Ammoniak hingegen macht das Band verschwinden und lässt nur eine schwache diffuse Absorption zwischen Grün und Blau, aber auf Zusatz von Säuren kehrt mit der röthlichen Farbe das schwarze Band zurück. 148

With the preparation of the zinc salt of hydrobilirubin from aqueous NH_3, spectrum analysis of the rose-red solution showed extinction from *Fraunhofer* line b (5183 Å) to the middle of the spectral range between b and F (4861 Å) – signifying a rather sharp band or narrow absorption region (*175*):

[148] Hingegen geben die ammoniakalischen Lösung des Farbstoffs, wenn sie etwas eines Zinksalzes (auch Cadmium) gelöst enthalten, besonders schöne Bänder. Es genügt, der stark ammoniakalisch gemachten Hydrobilirubinlösung ein paar Tropfen von Zinkchlorür oder –Sulfat hinzuzusetzen (wobei sich der entstandene Niederschlag leicht wieder löst) und diese Flüssigkeit vor den Apparat zu bringen. Oder man löst ausgefälltes Hydrobilirubinzink in Ammoniak und verdünnt. Beide Flüssigkeiten sind rosenroth und geben ein durch Schärfe und Dunkelheit ausgezeichnetes Band, das gegenüber den sauren Lösungen etwas nach links gerückt erscheint, daselbst bei 142 meiner Skale, also etwas vor b, scharf abgegrenzt, nach rechts hin verschieden breit ist, je nach der Concentration der Lösung, das aber immer am Dunkelsten von 142 bis 155 erscheint, d. i. von b an bis zur Mitte des Spectralabschnittes b bis F. Die ganze Erscheinung ist mindestens eben so empfindlich als die der sauren Pigmentlösung. 149

These data were to be compared to *Jaffe*'s urobilin pigment found in strongly-colored urine of feverish individuals said, as a dilute solution, to give a "dark shadow" from *Fraunhofer* lines b to F in the spectrum analysis (*185*):

Bringt man eine concentrirte Lösung vor den Spalt der Spectralapparate, so erscheint das Spectrum vom violetten Ende her bis etwas zur Linie b völlig dunkel; beim Verdünnen hellt sich der verdunkelte Theil allmählich auf und es bleibt schliesslich ein Absorptionsstreif

(γ) mit etwas verschwommenen Rändern an der oft genannten Stelle zwischen den Fraunhofer'schen Linien b und F.…

Die Verdünnung, bei der die Fluorescenz in Urobilinlösungen erscheint, ist enorm. Lösungen, die im durchfallenden Lichte fast farblos sind, zeigen im auffallenden noch deutlich grünen Schimmer, namentlich wenn sie den directen Sonnenstrahlen ausgesetzt werden. 150

Like hydrobilirubin, urobilin also fluoresced intensely as its zinc salt, and its absorption band, apparently much sharper than urobilin itself, lay between b and F, but closer to b than F (*185*):

Dieses Absorptionsband liegt, wie bereits angegeben . . . , zwischen den Linien b und F, aber der Linie b näher, als der Streifen der sauren Lösung (γ). – Es ist weit dunkler, schärfer begrenzt, als letzteres und bleibt noch bei den grössten Verdünnungsgraden sichtbar. 151

From the spectrum analyses of both hydrobilirubin and urobilin, *Maly* concluded in 1872 that they were identical. He was not again to publish results involving spectrum analysis until 1876, when he reinvestigated and clarified the reaction of bilirubin with Br_2 to give biliverdin, as he thought earlier, and a new blue pigment that he analyzed as the tribromo derivative (*177, 178*).

Maly's colorimetric spectral analysis of his bromobilirubin came to him courtesy of *von Vierodt*,[54] who during 1870–1881 modified and improved the *Bunsen-Kirchhoff-von Steinheil* spectroscope to incorporate a double collimator with adjustable slits used to calibrate the absorption of a sample to that of a reference, and used it to perform qualitative and quantitative studies of pigments in blood, bile, and urine. His instrument became a "standard" for nearly two decades (*184*). A dilute alcohol solution of bromobilirubin, which was blue, was found to transmit only green and blue light. With added NH_3 and a little $ZnCl_2$, the solution became grass-green, and its absorption spectrum showed two narrow, well-separated lines between 105 and 111 on a scale where Na is 120 (the Na *Fraunhofer* line is at 5892 Å) and Li is 102.5 (the Liβ line is at 6102 Å) (not the *Bunsen-Kirchhoff* scale), or exactly to the right of C, which corresponds to 6562 Å.

Of course, absent a quantitative characteristic such as the molar absorptivity (molar extinction) constant (ε) of the *Beer-Lambert* law and the wavelength at maximum absorption and bandwidth, the data from spectrum analysis were only marginally useful. Yet they furnished a potentially useful new characteristic for classifying or comparing bile pigments – and this spectroscopic method, like all others, would become better developed instrumentally, more exact in defining absorption characteristics, and more widely used. *Thudichum* also used the technique at about the same time as *Maly*.

In 1872 *Thudichum* published a *Manual of Chemical Physiology* in which he described experimental procedures for separating the components of bile and gallstones, including bilifuscin (probably $C_9H_{11}NO_3$) and bilirubin (or *Cholephäin*,

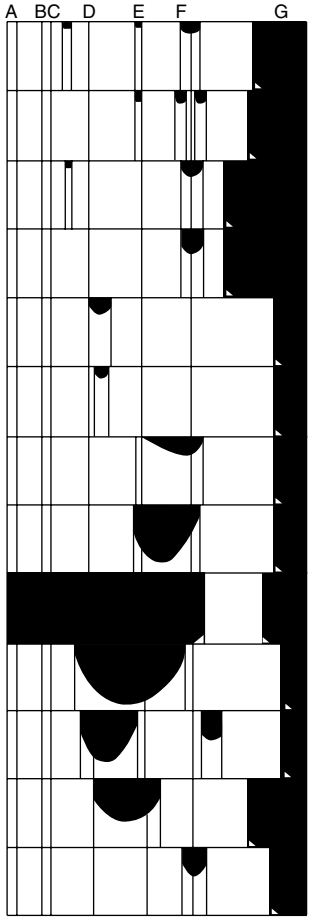
[54] *Karl von Vierodt* was born July 1, 1818 in Lahr, Baden and died on November 22, 1884 in Tübingen. He became Dr. med., a. o. Professor of theoretical medicine at the University of Tübingen in 1849, and in 1855 o. Professor and chair of physiology.

$C_9H_9NO_2$), from human gallstones. From the latter "in caustic or carbonated alkali exposed to the air for some days" (*186*), he prepared biliverdin ($C_8H_9NO_2$). And to an aq. NH_3 solution of bilirubin, he prepared blue *Cholecyanin* by adding conc. HNO_3 dropwise; whereas treatment of the solid directly with fuming or conc. H_2SO_4 led to the formation of green *Cholethalline*, *inter alia*. *Thudichum* provided spectra for each. It is unclear whether the spectra contain any information of diagnostic use, except that both samples are seen to absorb visible light strongly in the violet-blue region, and *Cholethalline* also absorbs strongly in the violet-green region.

Three years later, *Thudichum* published 13 spectra on the first page of a paper read before *The Chemical Society* and published in the May 1875 issue of the *Journal of the Chemical Society* (*158*):

Spectra referred to in this paper as diagnostic of certain Educts and Products.

1. Zn precipitate (Jaffé's), from rheumatic fever urine. Dissolved in alcohol and H_2SO_4. Colour, yellowish-red.
2. Urerythrine, from brick-red deposit in rheumatic fever urine. Dissolved in absolute alcohol. Colour, fiery-red.
3. Product by H_2SO_4 from No. 4. Dissolved in ether. Colour of solution, red.
4. Urochrome, normal; by H_2SO_4 from lead precipitate. Dissolved in water and acid. Colour, yellow.
5. Omicholine by H_2SO_4 from extract of urine. Dissolved in ether. Colour, red. Fluoresces green.
6. Omicholic acid; accompanies 5; soluble in NH_3. Dissolved in ether. Behaves like 5.
7. Uropittin, from extract or urine and urochrome by H_2SO_4. Dissolved in alcohol. Colour of solution, red.
8. Hydrobilirubin (Maly's), from bilirubin by Na amalgam. Dissolved in alcohol. Colour of solution, red.
9. Bromo-bilirubin in alcohol and HBr. Colour of solution, deep blue.
10. Same as 9, more dilute. Colour, fine blue.
11. Bromo-bilirubin changed by BaH_2O_2 and HCl. Colour, rose-red.
12. Sulphate of bromo-bilirubin in alcohol. Colour, violet-blue.
13. The same as 12, changed by hyposulphite and HCl.



The various intensities of absorption observed are expressed by shadows between perpendiculars, in tenths of the entire height of each spectrum. The rationality of the distances of the spectral lines is the empirical one of the author's spectrometer, described on p. 192 *et seq*. of the 10*th Report of the Medical Office of the Privy Council*. 1867. [Redrawn from ref (*158*)].

The work illustrates that by 1875 spectrum analysis was becoming widely adopted and in *Thudichum*'s lab served to distinguish: (i) a variety of urinary pigments from each other and from *Maly*'s hydrobilirubin, and (ii) his blue, brominated bilirubin from these pigments and its reaction products. *Thudichum*'s blue pigment, a dibromo-bilirubin which he felt certain had the formula $C_9H_7Br_2NO_2$, was formed by exposing a weighed quantity of dry bilirubin in a watch glass to Br_2 vapor. When Br_2 uptake had ceased (the weight of the bilirubin had tripled and no longer changed), *Thudichum* determined the ratio of the increase in weight due to bromine to the original weight of the bilirubin and took it to be the same as the ratio of the atomic masses of $2 \times Br - 2 \times H_2$ (or 158) to the atomic mass of bilirubin, which was thereby determined as 162.4. Thus, *Thudichum* felt he had accomplished an experimental determination of the molecular weight of bilirubin as 162.4, or very close to the 163 deduced by all other of his experiments. Hence the $C_9H_7Br_2NO_2$ formula. This seems rather like an attempt to fit an experimental result to a previously determined molecular weight value. With the assumption of an uptake of 2 Br_2, the equation used would have predicted 1.1942 (increase due to bromine) : 1.2280 (weight of dried bilirubin) = 316 (4 Br – 4 H) : 325, or twice the molecular weight assigned by *Thudichum* and thus a formula $C_{18}H_{18}N_2O_4$, or close to *Maly*'s first proposed $C_{16}H_{18}N_2O_3$ (mol. wt. 286), but not the doubled formula. *Thudichum's* dibromo-bilirubin was violet with a golden luster.

2.11 Bilirubin Polemics of the 1870s

Thudichum took issue with *Maly*'s research, chiding him for the early (incorrect) belief that biliverdin was the amide of bilirubin (*150*, *164*, *165*), which *Maly* had recanted some seven years prior (*166*, *167*). More to the point of bromination, *Thudichum* took issue with *Maly*'s earlier belief that bilirubin was oxidized to biliverdin by Br_2 (*166*, *167*). This too *Maly* had corrected in a subsequent paper (*171, 172*). Yet, perhaps unaware of the correction, *Thudichum* wrote a pointed yet valid criticism (*158*):

> The change is explained as oxidation, and in absence of any proof whatever, a somewhat analogous reaction is adduced to make the assumption probable; a reaction, however, the nature of which is as unknown as that which it has been called to illustrate.
>
> In contact with bromine vapour and moist air, bilirubin perhaps turns green for an instant, namely as long as the orange powder is able to send yellow rays through the blue compound, which quickly covers its surface. But often as I have repeated the experiment, it has had the same result in moist as well as dry air; never has there been formed a matter or colour similar to biliverdin, but always the brominated products above described.
>
> Further, if the green colour produced in the chloroform solution of biliverdin by bromine had been due to biliverdin, the latter must have been precipitated, as it is insoluble in chloroform. The green colour, according to my explanation, was simply a mixture of the yellow of the original solution, with the blue of the brominated product. The dark blue when once obtained remains unaltered for weeks, a good proof of the difference of this reaction from that of Gmelin, in which the blue produced by nitrous acid is of the most transient nature. The spectroscopy easily shows that the two blues are due to entirely different chemical entities. Even the blues produced by nitrous nitric acid in different bile-colouring matters are different. Their different spectra were originally observed and described by me in 1866 and 1867, in the 9th and 10th *Report of the Medical Officer of the*

> *Privy Council*. See the latter volume, p. 251 to 260. Cholocyanine; its sulphate; sulphate of sulpho-cholocyanine; and hyocoerulin. Therefore in reactions with bile-colouring matters a blue colour is no more a proof of identity than a green.

Note *Thudichum*'s reference to spectroscopy as a means of distinguishing the blue bromination product from the blue pigment of the *Gmelin* reaction.

Maly's work was not alone in *Thudichum's* gun sight, and he did not spare any criticism of the *Bilicyanin* that *Heynsius* and *Campbell* obtained by what they called an oxidation of bilirubin by bromine water (*153*, *154*) or the greenish *Choleverdin* of *Stokvis* (who later declared it identical to *Bilicyanin*). To these gentlemen he issued a stern rebuke (*158*):

> A most elaborate account of the alleged oxidation-products of bile-pigments and their absorption-bands was published by A. Heynsius and J.F.F. Campbell, in *Pflüger's Arch. f. Physiol.*, iv, 497-547, extending over fifty pages. A blue substance, *bilicyanin*, was obtained by what is termed the oxidation of bilirubin by bromine-water. The spectra obtained varied, as also did the solubilities of the products. Not a single product was isolated, and none was analysed. It is easy to see that these products were principally mixtures of the mono- and dibrominated bilirubin. Of oxidation there is no evidence whatever. The same remarks apply to a greenish product, obtained formerly by Stokvis, and termed choleverdin, which, after perusal of the paper just quoted, he declared to be identical with and thenceforth termed bilicyanin (*Neues Report. f. d. Pharm*., 21, 732-737). Without entering into any detailed discussion of these discursive papers, which relate merely to experiments made with dilute impure solutions in test tubes, and do not start with any pure substance, nor arrive at any stoïchiometrical conclusion, I hope that the following conclusions will be acceptable to the reader.

And he clarified all of the alleged bromine-induced oxidations of bilirubin as nothing more than brominations (*158*):

> The allegation made by Maly, that bilirubin under the influence of bromine was converted into biliverdin is unfounded.
>
> The allegation made by Maly, Heynsius and Campbell, and Stockvis, that bilirubin under the influence of bromine yielded products of oxidation, is unfounded.
>
> The products obtained by his halogen are not products of oxidation but of substitution.

It was not just misinterpreted bromination reactions of bilirubin that attracted *Thudichum*'s attention and drew his response. For he also keyed in on *Maly*'s hydrobilirubin and *Maly*'s belief that he had transformed bilirubin into the coloring matter of urine, that there was a probable relationship to *Jaffe*'s urobilin (*158*):

> Maly (*Ann. Chem. Pharm*., 1872, No. 7, p. 77) claims to have transformed bilirubin into the colouring matter of urine, "at least," he says, qualifying considerably his general title, "that kind of urinary colouring matter which according to Jaffé, is the best defined." Now although Jaffé has extracted from urine, by means of zinc oxide, a mixture of at least two of the decomposition products of urochrome, and has described their spectral phenomena, long since and originally published by me, as if they belonged to a single body, and as if they were new discoveries, yet he has not isolated a single pure substance and has not instituted a single elementary analysis.
>
> At first sight, therefore, the metamorphosis announced by Maly was extremely improbable to any one acquainted with the chemical bearing, composition, and physical qualities of the bodies in question. But the spectroscopic identity of the products of Maly and Jaffe [sic] was announced with such assurance, that I felt it my duty to repeat some of the relative experiments of these authors.

Repeating *Maly*'s preparation of (reddish) hydrobilirubin by reduction of bilirubin using Na(Hg), *Thudichum* found the same product as *Maly*, unchanged from the first half of the reaction to one lasting two days. He disputed the identity of hydrobilirubin as neither urobilin nor urochrome based on the results of his spectrum analysis and, even better, by the fact that hydrobilirubin is insoluble in water whereas urochrome is soluble. (Urobilin was an educt, found only in the urine of feverish patients after standing. *Urochrome* was the term coined by *Thudichum* for the matter to which urine owed its yellow color; it was not the chromogen of urobilin.) Further investigation simply reaffirmed his conclusions. Not one to eschew analysis and criticism of hydrobilirubin, which *Maly* believed to be a tribasic acid, *Thudichum* also objected to *Maly*'s formula ($C_{32}H_{40}N_4O_7$) for it and the sparse supporting evidence: it formed only one silver compound that analyzed for 35.75% Ag, and one zinc compound that analyzed for 14.2% Zn; whereas other analyses of the former yielded 37.1% Ag and up to 37% Zn for the latter. He was very plainly unconvinced of its formula and remained emphatic that hydrobilirubin and *Jaffe*'s urobilin were not at all the same (*158*):

> These data therefore do not afford the means for determining either the atomic weight or the basicity of the new product, but seem to show that the sodium-reaction produces a variety of new products, which remain partly mixed in the precipitate, partly in the mother-liquor from which it falls. For this liquid remains red, and retains a considerable quantity of a by-product.

Thudichum further cast doubt on *Jaffe*'s urobilin, which was obtained by *Jaffe* only from pathologic urine, noting that the pigment had been diagnosed by *Jaffe* only by a spectroscope and was never isolated. In his attempt to isolate urobilin, *Thudichum* found that it separated into a mixture of urochrome and "urerythrin", the latter also found dissolved in fresh urine to which it imparted a reddish-yellow color. He refuted any similarity between these pigments and hyrobilirubin (*158*):

> The following are the *irreconcilable differences* between hydrobilirubin on the one side, and urochrome and all its products and urerythrin on the other side.
>
> Urochrom is yellow, soluble in water, shows narrow faint band in acid mixture, none in neutral or alkaline solution.
>
> Hydrobilirubin is brownish red, insoluble in water, easily soluble in watery acid, and in alcohol with deep red colour, and spectrum differing entirely from urochrom.
>
> Urochrom, when concentrated enough to show any band, is by boiling with acids immediately split up into omicholin, uropittin, and uromelanin, each of which products can be separated out and recognized with the greatest ease either spectroscopically or by chemical tests.
>
> Hydrobilirubin is not altered by boiling with acids in any characteristic manner; it is certainly not split up, and yields not one of the products of urochrome.
>
> Hydrobilirubin is, therefore, not identical with, or even similar to, the urinary colouring matters, including urerythrin. Its only similarity spectrally is to uropittin, but the general differences between the two bodies are striking.

And then he moved on to stating his objections to *Städeler*'s newly expressed hypothesis on the theory of bilirubin and its metal salts – apparently the last commentaries by *Städeler* before his death. It seems that *Städeler* had read of the many studies of bilirubin and its salts reported in 1868 (*152*), and he attempted to bring them into harmony with his own concepts of bilirubin, as revised to accommodate

the new data. It did not sit well with *Thudichum*. Although *Städeler*'s very early elemental combustion analyses of bilirubin found favor with *Thudichum*, who found it coincident with his own but at odds with *Städeler*'s new hypotheses – hypotheses that included the doubling of the bilirubin formula to $C_{32}H_{30}N_4O_6$ and assertion that it had six replaceable hydrogens to accommodate metal salt formation drew special ire (*158*):

> I do not believe that this hypothesis has any foundation in fact. Not a single formula of Städeler's, and not a single element of any formula, can be derived from my analyses. . . . … I hold the hypothesis of Städeler to be merely not proved by facts, but to be directly disproved by all my analyses, with exception . . .

Yet *Maly* was a special early whipping boy. An apparently exasperated *Thudichum* felt compelled in 1876 (*161*, *162*) to write "An open letter to the Imperial Academy of Sciences at Vienna, containing an examination of the researches on the colouring matter of bile, by *Richard Maly*, of Graz." This polemic was in response to what apparently became (or caused) *Maly*'s final publication on bile pigments in 1875 and is illustrated in the following excerpts from 14 of the 40 very detailed points of *Thudichum's Offenes Sendschreiben* (*162*):

> 8. That Prof. Maly had received the letter containing the foregoing passages is proved by the reply which he addressed to me, dated from Innsbruck, June 24 (1874), now before me. Prof. Maly, therefore, before he began the experiments which are so exhaustively described in the fifth paper, was not only informed of his error, but actually in possession of the key to his alleged discovery, and it was therefore impossible that he should have been led to this discovery by his experiments.
>
> 9. … That these researches and publications should have remained unknown to the editor of an annual report on the progress of animal chemistry is not impossible, but that he excluded the contents of my letter from the circumference of the "usual duty" admits of only *one* explanation, but not of justification.
>
> 10. … This description leaves the main points which have been established by my researches entirely out of consideration, and in all particular statements it is completely incorrect. Indeed I can hardly believe that Prof. Maly has read my paper; it is certain he has not understood it.
>
> 16. … All these necessary precautions Prof. Maly has neglected, and in consequence has arrived at conclusions which have no foundation.
>
> 17. … Prof. Maly further endeavors to influence the judgment of the Academy by raising doubts in general regarding my experiments; first, on the ground that I had performed each experiment only once; secondly, because I had not analysed the final product. Against these objections I maintain that the above experiment, considered by the light of my former researches in the *Journ. d. Pract. Chem.* (civ., 193), requires no further analysis. I thought and think every analysis of the product to be a mere waste of time, – every repetition on my part a waste of labour and material. However, in order to meet the object, and from a high regard for the Academy, I have repeated the experiment described under 15, yet two several times, and have analysed the products by determining quantitatively the amounts of carbon, hydrogen, nitrogen, and bromine. . .
>
> 21. … In making this statement Prof. Maly loses sight of "the usual duty of characterising previous knowledge," or other knowledge. . . .
>
> 22. … In the letter alluded to, Städeler, in view of my researches, abandons all his former formulæ, and coerces my results by an utterly unjustifiable process of re-calculation, in which no single analytical result harmonises with the new hypothesis into some sort of support for his doubled formula and hexa-basic acid hypothesis, without having produced a single compound or made a single new analysis.

23. Prof. Maly causes to himself many difficulties by his preconceived opinions and uncontrolled imagination, as I am obliged to prove now more in particular. . . .

25. … How can an author who works with such preparations call others to account for the alleged impurity of their preparations!

27. The Academy may justly demand of me to prove these statements. I am ready, on receiving a request to that effect, to communicate to the Academy details, the extent of which are excluded from the present letter on account of their length. . . .

30. … On the contrary, it must be maintained that such results and corollaries are directly opposed to the principles of chemical science, and slap the endeavor for final accuracy rudely upon the face.

32. The observation of the influence of sodium amalgam upon bilirubin, which led Prof. Maly to the discovery of the so-called hydro-bilirubin, would have been an interesting progress in our knowledge concerning bilirubin. But as the author starts from erroneous views regarding the composition and molecular weight of bilirubin, his conclusions regarding his product and its composition, and regarding the formula of the change, are necessarily erroneous. . . .

39. It is impossible here to point out all the irrelevant and erroneous detail with which Prof. Maly surrounds his faulty observations. . . .

40. I conclude my letter to the Imperial Academy with the expression of the deepest regret concerning the circumstances which have compelled me to write it. I should not be able nor dare to molest the Academy a second time with this matter, and I therefore pray the Academy to excuse the length and serious tone of this letter, with the importance which the matter has for me, for science, and for the maintenance of the ethical rules which govern the intercourse or cultivators of science. I hope that the Academy will give to my letter no less publicity than it has given to the papers which have called it forth.

2.12 Conjectural Chemistry and Bilirubin Polemics at the Close of the 19th Century

The last quarter of the 19th century brought new investigators into the bile pigment field, most with medical-physiological interests and sophistication but with an incomplete understanding or knowledge of the earlier chemical studies and errors therein. Even as he neared the sunset of his long life, *Thudichum* had not abandoned his penchant for "setting the record straight", while apparently retaining his intellectual vigor and keen memory. Three-quarters of the way into the 19th century he believed he had settled some of the important problems associated with bilirubin, its purification, combustion analysis, controversial formula, "spectrum analysis" characteristics, the controversy with *Maly* over the reaction products with Br_2 and even the non-equivalence of its Na(Hg) reduction product (*Maly*'s hydrobilirubin) with urobilin, *Jaffe*'s purported urinary pigment – that *Thudichum* had discounted as such, *etc*. So 20 years later, after having turned his interests over to research on brain chemistry during the previous two-plus decades, it must have come as something of a shock to him to discover that the error-laden publications of others in the 1870s were being cited to support work in the 1890s.

Thudichum responded forcefully in print, from 1896 to 1899 (*187–190*), by pointedly citing where and how the authors had been led astray by earlier errors (especially *Maly*'s) – that the new authors were basing their work on the *conjecture* of

others, unsupported by high quality experiments. In apparent exasperation with the extent to which errors had permeated the literature and were being promulgated uncritically, in 1900 he coined the word *Conjecturalchemie* (conjectural chemistry) (*191*) – a term he used to chastise researchers for the propagation of their (defective) conclusions or statements based on *supposition*, not fact, and previously found (by *Thudichum*) to be deficient of firm experimental verification. Thus *Thudichum* responded forcefully to a long, comprehensive article in 1893 by *F. Grimm*, a physician in Berlin, on the urobilin of normal patients and those with a wide variety of pathologies (*192*). *Grimm*'s article, which contained references to the studies of *Jaffe*, *Maly*, *Hoppe-Seyler*, and others, but no reference to *Thudichum*'s earlier work on the pigments of urine summarized in his treatise on the same (*193*) clearly provoked *Thudichum*. In 1897, he indicated that when he referred to urobilin it was the pigment isolated from urine by *Jaffe*'s process; whereas, most of the later reports on urobilin related to the product obtained by a different process. On this basis, he stated that the pigment isolated from human feces is not urobilin but the intestinal *Lutein* that he had reported earlier and was in no way identical to the compound obtained from urine – and all reports on its identity (with urobilin) were in error (*188*):

> Also ist dieser Körper in den Fäces, der übrigens nie isolirt worden ist, von dem aus Harn gänzlich verschieden, und alle Angaben über Identität u. s. w. sind irrthümlich. 152

Thudichum again did not hesitate to reprimand (the deceased, 1891) *Maly* for having indicated that *Jaffe's* urobilin was identical to his hydrobilirubin obtained from Na(Hg) reduction of bilirubin. He declared it absolutely erroneous: "Auch dies ist ein absoluter Irrthum" (*188*). Then he proceeded to critique *Grimm*'s work, which used mainly spectrum analysis to correlate urobilin with the various pigments that he had isolated from urine, for assuming that hydrobilirubin and urobilin were identical, and for naming the product from urine hydrobilirubin. But 14–20 years earlier *Thudichum* had proven them not to be identical, saying that the proof is entirely indisputable, and thus cannot even be contested: "Der Beweis ist ganz unanfechtbar, und daher auch nicht angefochten worden" (*188*). He declared that using the name *hydrobilirubin* for any product of urine, that hypotheses from that related to the transformation of bilirubin, and that physiological and pathological speculation based on it were absolutely in error. And he decried the use of spectrum analysis as the only means of identification, *etc*. However, shortly thereafter, in 1898 (*189*) *Thudichum* had to be pleased with the proof, based on the combustion analysis by *Hopkins*[55] and *Garrod*[56] (*189*).

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[55] *Sir Frederick Gowland Hopkins* was born on June 20, 1861 in Eastbourne, Sussex, and died on May 16, 1947 in Cambridge, UK. He taught physiology and toxicology at Guy's Hospital, London, from 1894-1898, became Reader in chemical physiology at Cambridge University from 1902-1914, then professor from 1914, and in 1929 was awarded the *Nobel* Prize in Physiology or Medicine (with *Christian Eijkman*) for the discovery of vitamins.

[56] *Sir Archibald Edward Garrod* was born on November 25, 1857 in London, and died on March 28, 1936 in Cambridge, UK. He was a physician who saw dynamic biochemistry in metabolic pathways, and recognized Mendelian heredity as an explanation for inborn errors of metabolism (albinism, alkaptonuria, cystinurea, and pentosurea).

Hopkins and *Garrod*'s analyses (*194*) based on *Maly*'s hydrobilirubin and urobilin isolated from various human sources: normal and pathological urine, feces, and bile from the post-mortem gallbladder, although resembling each other in certain properties, were very clearly different in the %N (*194*):

SUMMARY OF RESULTS.

	Urinary Products				Fecal Products	
	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2
C	63·69	–	–	63·24	–	63·81
H	7·73	–	–	7·60	–	8·20
N	4·02	4·22	4·05	4·09	4·17	–

	Urobilin	Hydrobilirubin		
	Mean of above results	Theory	Mean of Maly's results	Our estimation of nitrogen in hydrobilirubin
C	63·58	64·86	64·68	–
H	7·84	6·75	6·93	–
N	4·11	9·45	9·22	9·57
O	24·47	18·94	19·17	–

[Where the urinary products are from: No. 1, a patient with hepatic cirrhosis; No. 2, a patient with pernicious anemia; No. 3, a patient with intestinal obstruction; No. 4, mixed urines of hospital patients in surgical wards. And the fecal products are from: No. 1, stools of a case of typhoid fever in the early convalescent stage; No. 2, normal feces].

Despite an expressed "uncertainty with regard to the question of ash", the clear difference between *Maly*'s hydrobilirubin and natural urobilin, *Hopkins* and *Garrod* still held the belief that they shared a relationship (*194*):

> We may be permitted to say that we entered upon the analysis of urobilin obtained from natural sources in the hope that our results might help to place upon a firmer foundation the belief, which has prevailed since the publication of Maly's results, that there exists a *simple* relationship between that pigment and bilirubin. This hope has not been justified by the results, and we are convinced that the relationship is by no means so simple as has been supposed. The change from bilirubin to urobilin cannot be a mere question of reduction and hydrolysis, but must necessarily be attended by a removal of nitrogen; of this our analyses leave no doubt whatever.
>
> On the other hand we cannot doubt that the one pigment is actually derived from the other, a conclusion which evidence of other kinds appears to us to render unavoidable.

The data also pointed to *Hopkins* and *Garrod*'s conclusion that the urinary and fecal urobilins are identical. *Thudichum* had earlier (*193*) objected to this, a then unproven prospect, and broadened his 1898 report on urobilin to include comments on his urinary urochrome, *Omnicholin*, *Urorhodin*, and *Uropittin*. He commented that *Hopkins* and *Garrod* had not come up with a formula for their analysis of urobilin (they said they did not feel themselves in a position to attempt to assign an

empirical formula (*194*, *195*)) – but *Thudichum* was less inhibited and gave $C_{18}H_{25}NO_5$ (*189*). In fact, *Hopkins* and *Garrod* indicated that (*194*):

> The figures obtained do not appear to lend themselves to a formula showing any simple relationship to that accepted for bilirubin, and until experiment has shown by what chemical steps a product strictly agreeing in its general characters with natural urobilin can be prepared from bile pigment it is undesirable to pursue the question of its constitution.

Hopkins and *Garrod* brought forth (*194*) several interesting points related to bilirubin metabolism. By allowing Na(Hg) to act upon bilirubin beyond the stage specified by *Maly*, the product resembled natural urobilin more closely (*194*):

> Passing on to the consideration of the further question we may say at once that the results which we have obtained by allowing the action of sodium amalgam to proceed further agree closely with those of Disqué and Eichholz. As the action proceeds the liquid assumes a pale yellow colour, the extra alkaline bands disappear and the precipitability of the urobilin-like product by hydrochloric acid is conspicuously diminished. When acidified, filtered and exposed to the air the liquid darkens and the absorption band gains in intensity. The product so obtained bears a far closer resemblance to the natural pigment than Maly's hydrobilirubin does.

And of seemingly greater importance, because it almost certainly showed a relationship between bilirubin as a metabolic precursor to urobilin, as is understood today (*194*):

> It is a well-known fact that in health the bile pigment which enters the duodenum disappears, as such, before the intestinal contents are expelled, and in its place we find in the fæces urobilin and its chromogen.
>
> When, as in certain cases of typhoid fever, the bile pigment is found in abundance in the fæces, the urobilin is greatly diminished in quantity or altogether wanting. When the flow of bile into the intestine is arrested urobilin and its chromogen disappear from the fæces, to reappear when the patency of the bile ducts is re-established.
>
> Friedrich Müller . . . has further shown that when bile is introduced into the stomach of a patient with complete biliary obstruction and whose fæces are urobilin-free, urobilin appears in the stools.

Thudichum had less patience with the 1894 publication of *Jolles*[57] on the oxidation of bilirubin to biliverdin using I_2 (*196*). Apparently the lessons associated with the *Thudichum-Maly* (one-sided) polemics of the 1870s had become unlearned by the last decade of the 19th century, a time when there appeared a renewed interest in bilirubin and related pigments. In 1894, *Jolles* published a very long paper on a quantitative method for determining bilirubin in bile using I_2 as an oxidant (*187*). In this work, he described, *inter alia*, his study of the oxidation of bilirubin to biliverdin using a dilute alcoholic solution of I_2, for which he

[57] *Adolf Jolles* was born on November 9, 1862 in Warsaw and died on November 13, 1942 in Theresienstadt. He was an Austrian chemist and in 1894 a young docent at the k.k. technologischen Gewerbsmuseum in Vienna. *Jolles* had a long and productive career as an analytical/medicinal chemist in Vienna with numerous publications that brought recognition for his urinary tests (determining bile pigments and albumin in urine), detecting "hematoporphyrin" in the urine of patients with drug-exacerbated porphyria, his studies of fats, and a test for pigments (*Jolles*' test), *etc*.

found precedent in *Maly*'s published oxidation by halogens. *Jolles* wrote a chemical equation describing the interconversion of *Maly*'s and *Städeler*'s formulas for bilirubin and biliverdin (*196*):

$$C_{32}H_{36}N_4O_6 + 4\ J + 2\ H_2O = C_{32}H_{36}N_4O_8 + 4\ HJ$$

The work caught the eye of *Thudichum*, who despite his advanced age and having earlier redirected his research to a study of the chemical constituents of the brain, must have been surprised or perhaps even shocked to realize that his important studies on the halogenation of bilirubin had gone unread or unappreciated and that his rejection of the *Maly* and *Städeler* formulas had gone unrecognized. After repeating *Jolles*' I_2 reaction in $CHCl_3$ and with I_2 vapor and finding no reaction or no biliverdin among the reaction products, but also providing scant experimental details, he issued a stern rebuke in 1896 (*187*). The complaint was that *Jolles* based his work on the disproven results of *Maly*, published in 1868 (*166*, *167*), that bilirubin is oxidized to biliverdin by Br_2, which was proven by *Thudichum* to be a substitution reaction (hydrogen for bromine) some 20 years earlier. He chastised *Jolles* for overlooking *Maly*'s correction in 1875 (*171*, *172*), where, prompted by *Thudichum*, *Maly* had conceded that the reaction of bilirubin with Br_2 was not an oxidation but a substitution. He objected to *Jolles*' not having isolated or analyzed the reaction products (*187*):

> Der Aufsatz des Hrn. Jolles beginnt mit der ganz unbedingten Angabe, dass das Bilirubin durch (eine alkoholische Lösung von) Jod in Biliverdin verwandelt werde. Diese Behauptung ist indessen nur eine aus der angeblichen und widerlegten Reaction des Broms hergeleiteten Analogie und fällt deshalb mit ihrem Muster. Es ist auch gar kein Versuch gemacht, die angebliche Reaction zu begründen, es sind keinerlei Produkte isolirt oder analysirt worden. Ich könnte mich daher mit dem Resultat begnügen, dass, da die Prämissen des Hrn. Jolles gar nicht existiren, seine Thesen nothwendiger Weise dasselbe Schicksal haben. 153

Thudichum went further, with point by point admonishments directed toward *Jolles*' published work. He objected to *Jolles*' spectroscopic characterization of biliverdin as the product of I_2-promoted oxidation of bilirubin, finding a mismatch between *Jolles*' product and authentic biliverdin (*192*):

> Spectroskopische Angaben. Auf S. 3 seiner Abhandlung sagt Hr. Jolles, er habe die Identität seines grünen Produkts, aus Galle oder Bilirubin durch Jod erhalten, mit reinem Biliverdin spectroskopisch bewiesen. Nun hat aber das aus Bilirubin durch Einfluss von Soda als Lösungsmittel und Luft als Oxydationsmittel dargestellte Biliverdin keine specifischen Absorptionsschatten in seinem Spectrum. Daraus allein folgt, dass, da Jolles' grüne Produkte, durch Jod aus Galle oder Bilirubin erhalten, solche Absorptionen zeigen, diese Produkte nicht mit Biliverdin identisch sind. 154

He objected to *Jolles*' use of unproven formulas and especially for an unproven reaction, bilirubin + I_2 " biliverdin (*187*):

> Unbegründete Formeln. Damit verschwindet die in der Abhandlung verschiedentlich wiederholte Formel, wonach eine Molekül sogenannten Bilirubins 4 Atome Jod und zwei Molekül Wasser zur Oxydation zu Biliverdin erfordern und aufnehmen solle. Da die Reaction überhaupt nicht existirt, so müssen die den Pigmenten zugeschriebenen Formeln ungültig sein. 155

Thudichum was especially angered that *Jolles* would use a bilirubin formula ($C_{32}H_{36}N_4O_6$) ascribed to *Maly* and *Städeler* because, as he stated, *Maly* had determined no formula for the pigment, had not once completely analyzed (by combustion) the pigment – and especially the %N had not been determined or weighed. *Städeler* had determined a different formula ($C_{16}H_{18}N_2O_3$) to bilirubin then doubled it in 1870 in order to establish it as a hexabasic acid, which it is not. *Thudichum* had no kind words on this sore point (*187*):

> Herr Jolles wiederholt die irrige Angabe, Maly und Städeler hätten die Formel des Bilirubins als $C_{32}H_{36}N_4O_6$ „bestimmt". Allein Maly hat überhaupt keine Formel für Bilirubin bestimmt; er hat es nicht einmal vollständig analysirt, und insbesondere den Stickstoff seines Präparats weder gemessen noch gewogen. Er war daher gar nicht in der Lage, eine Formel zu berechnen. Nur Städeler hatte dem Bilirubin die Formel $C_{16}H_{18}N_2O_3$ beigelegt, dieselbe aber um 1870 verdoppelt, um dasselbe als eine sechsbasiche Säure darstellen zu können. Dieser ganz ungerechtfertigte Versuch ist vollständig misslungen. 156

Thudichum continued, unrelentingly, taking *Jolles* to task on (i) the latter's assertion that cattle bile contains no bilirubin; (ii) the latter's spectra of bilirubin and biliverdin and failure to recognize that he (*Thudichum*) had studied the reaction of bilirubin with I_2 and Br_2 much earlier; (iii) *Jolles*' belief that *Choletelin* is the end-product of the reaction of bilirubin with Br_2, which *Thudichum* claimed to have shown to be invalid and that choletelin came from reaction with HNO_2 and not from Br_2; (iv) *Jolles*' stating incorrectly that bile contains lecithin and making incorrect comments on urobilin, which *Thudichum* said was long ago disproved – ox bile contains no lecithin but a phosphatide with four nitrogen atoms, and the urobilin statements (that swine bile contains relatively high amounts) were completely refuted in 1875.

Using his 1896 publication (*187*) as a vehicle for more corrections, *Thudichum* did not fail to remind that the Italian Professor *Capranica* incorrectly stated that a $CHCl_3$ solution of bilirubin gave biliverdin upon exposure to sunlight, that it gave only chlorinated products. (This, however, was later shown to be untrue; the reaction does in fact yield some biliverdin.) Not one to let "sleeping dogs lie", *Thudichum* resurrected the ancient history of how *Städeler* reached his formulas for bilirubin and explains for *Jolles'* benefit why they are incorrect and indirectly admonished him for not having recognized it. *Thudichum* accepted *Städeler*'s earliest bilirubin formula ($C_9H_9NO_2$) as the only correct version. It matched his own. He disavowed the later *Städeler* formula ($C_{16}H_{18}N_2O_3$), which was based on the neutral calcium salt of bilirubin ($C_{32}H_{34}N_4O_6$, from $2(C_{16}H_{17}N_2O_3)$ + Ca) and what *Thudichum* described as its questionable calcium determination of 9.1% Ca. From his own studies of bilirubin calcium salts, in which he found both a neutral salt and a half acid salt, *Thudichum* cited that the latter ($C_{27}H_{29}N_3O_8Ca$, based on *Städeler*'s $C_9H_9NO_2$ for 3 × bilirubin + $Ca(OH)_2$) theoretically has 7.1% Ca (*Städeler* found 6.5%); in contrast the neutral salt ($C_{18}H_{20}N_2O_6Ca$) yields 10% Ca – and thus cannot be identical to *Städeler*'s. *Thudichum* claimed that after his own investigations were published *Städeler* gave up on the second bilirubin formula in favor of its doubled formula ($C_{32}H_{36}N_2O_6$) without carrying out a single experiment or analysis. He noted that there is no correlation between the last *Städeler* formula and his

preparation and analysis and attributed *Städeler*'s changing formulas to desperation and the result of following an incorrect calcium analysis (*187*):

> Nach der Veröffentlichung meiner Untersuchungen nun gab Städeler auch diese zweite Formulirung des Bilirubins auf, und damit natürlich alle anderen Formeln seines Biliverdins, Biliprasins und Bilfuscins, verdoppelte seine contrahirte Formel für Bilirubin zum zweiten Mal, auf $C_{32}H_{36}N_4O_6$, und erklärte es für eine sechsbasische Säure; dazu hatte ihn vielleicht die damals eben gemachte Entdeckung der Honigsteinsäure verleitet. In diese Hypothese suchte er nun meine Resultate einzuzwängen, ohne eine einzige Darstellung oder Analyse auszuführen. Keine einzige der Formeln Städeler's und kein einziges Element irgend einer seiner Formeln kann aus meinen Präparaten und Analysen abgeleitet werden. Die von ihm berechneten Metallmengen der Verbindungen betragen alle von 1% bis zu 6% weniger als die von mir bestimmten Mengen. Ich kann dieses Verfahren Städeler's nur als ein Resultat der Verzweiflung an allen seinen Arbeiten bezeichnen. Nach meiner Ueberzeugung waren seine ersten Präparate rein und sein Analysen richtig, obwohl er sie selbst, ohne Erklärung, irriger Weise verleitet durch eine trügerische Kalkbestimmung, aufgegeben hat. 157

Unlike *Maly*, who seems to have been chased out of the bilirubin arena by *Thudichum*'s forceful dismantling of his work, *Jolles* did not back down and in 1899 published a polite but assertive rejoinder (*197*). In this he focused almost exclusively on *Thudichum*'s main point: that treatment of bilirubin with iodine could not lead to an oxidation of the pigment (to biliverdin) but caused only a substitution reaction. He expressed astonishment that *Thudichum* had not carefully read his earlier paper of 1894 (*196*). For *Jolles* insisted that he had not (as *Thudichum* wrote) cited *Maly*'s work on the oxidation of bilirubin to biliverdin, published in 1868, in support of his own work, and that he also had not failed to recognized *Maly*'s revocation, in 1872, of the 1868 work which suggested that altered reaction conditions could lead to different results/products (*197*):

> Bevor ich auf den sachlichen Inhalt der Einwendungen des Herrn Thudichum näher eingehe, muss ich zunächst meinem Erstaunen darüber Ausdruck geben, dass Thudichum meine Abhandlung anscheinend nur ganz flüchtig durchgelesen hat, indem er ganz willkürlich und unberechtigt von meiner Arbeit angiebt, dass sie auf die angebliche Entdeckung des Prof. Maly aus dem Jahre 1868, wonach das Bilirubin durch Brom vermittelst eines Oxydationsprocesses in „Biliverdin" verwandelt werden sollte, basire. Diese Unterschiebung muss ich aber mit Entschiedenheit zurückweisen, denn ich habe die Untersuchungsergebnisse Maly's nicht als Beweismittel für die Richtigkeit meiner Resultate herangezogen, sondern ich habe nur – wie üblich – in der Literatur, soweit dieselbe meine Arbeit zu tangiren schien, die von Maly im Jahre 1868 publicirten Ergebnisse anzuführen mich für verpflichtet gehalten. Herr Thudichem irrt sehr, wenn er glaubt, dass mir die zweite Arbeit Maly's vom Jahre 1872, in welcher er die erste Arbeit widerruft, nicht bekannt war. Aber der Umstand, dass Maly bei seiner zweiten Arbeit ganz andere Versuchsbedingungen eingehalten hat, die für die Entstehung von bromirten Substitutionsprodukten günstiger gewählt waren, und es als selbstverständlich vorausgesetzt werden kann, dass die Aenderung der Versuchsbedingungen gerade bei der Einwirkung von Halogenen zu ganz anderen Ergebnissen führen könne, sowie andererseits der Umstand … 158

To *Jolle*s, the issue was not *Städeler*'s formulas or *Maly*'s or *Thudichum*'s, and it was certainly not the bromination reaction investigated by the latter two; it was *Thudichum*'s insistence that *Jolles*' oxidation of bilirubin to biliverdin by I_2 could

not happen. In rebuttal, *Jolles* indicated that the results from treating bilirubin with Br_2 was a poor model for reaction with I_2, that he had shown that, in dilute solutions, a molecule of bilirubin reacted with four atoms of iodine to produce a green pigment that he characterized as biliverdin – and that process could be used to detect bilirubin in animal bile and (quantitatively) in urine, which was the reason for his original study (*197*):

> … dass ich in meiner Arbeit ja keinen anderen Zweck verfolgt habe, als eine Methode bekanntzugeben, welche gestattet, das in den thierischen Gallen enthaltene Bilirubin quantitativ zu bestimmen, hat es mir als überflüssig erscheinen lassen, auf die zweite Maly'sche Arbeit in dem Litteraturverzeichnisse hinzuweisen. Die Quintessenz meiner Arbeit war ja doch nur die, zu zeigen, dass bei Einhaltung bestimmter Versuchsbedingungen Bilirubin quantitativ in einen grünen Farbstoff übergeführt wird, wobei auf 1 Mol. Bilirubin 4 Atome Jod verbraucht werden, so dass dieser Process ein Mittel an die Hand giebt, den Bilirubingehalt in den thierischen Gallen und in Harnen quantitativ zu bestimmen. Dass diese meine Methode den Zweck erfüllt, beweisen meine zahlreichen Beleg-Analysen, die bisher noch Niemand widerlegt hat, und thatsächlich haben bereits anerkannte Handbücher, wie Huppert in der 10. Auflage seiner Anleitung zur qualitativen und quantitativen Analyse des Harns (S. 865) die Methode zur annähernden quantitativen Bestimmung des Bilirubins im Harne empfohlen. 159

Thudichum viewed the green pigment as simply an iodinated substitution product of bilirubin, much as the reaction with Br_2 gave bromine substitution; *Jolles* disputed the first statement, not the last, and stood firm on the reaction with I_2 being an oxidation (to biliverdin). He acknowledged not having proved that the green product is actually biliverdin, but in the 1899 publication he gave full details (*197*):

> Thudichum den grünen Farbstoff als ein jodirtes Substitutionsprodukt, ich jedoch als ein Oxydations-produkt, und zwar als Biliverdin, ansehen. Ich gebe gern zu, dass Thudichum insofern Recht hat, als ich für die Identität des grünen Farbstoffes mit Biliverdin die analytischen Belege nicht geliefert habe. Jedoch bemerke ich, dass mir schon damals ein genügendes noch nicht zur Publikation gebrachtes Material vorlag, auf Grund dessen ich mich berechtigt hielt, den bei der Einwirkung einer verdünnten alkoholischen Jodlösung auf Bilirubin vor sich gehenden Process als eine Oxydation anzusehen und den hierbei zunächst entstehenden grünen Farbstoff als Biliverdin anzusprechen. 160

Jolles thus focused on *Thudichum*'s dogma that I_2 cannot cause oxidation of bilirubin, only substitution (*197*):

> Was nun Herr Thudichum in erster Linie bestreitet, ist die Thatsache, dass bei der Einwirkung der Jodlösung auf gelöstes Bilirubin eine Oxydation vor sich gehe, es könne sich nach ihm einzig und allein nur um einen Substitutionsprocess handeln. . . . Herr Thudichum stellt sich in seiner Erwiderung auf den eigentümlichen Standpunkt, dass alle seine Behauptungen bezüglich der Gallenfarbstoffe förmlich als unumstössliche Dogmen zu betrachten wären. 161

Jolles thus asked a fundamental question: why could *Thudichum* not see the possibility of several competing reactions taking place by reaction of bilirubin with I_2 (or Br_2 for that matter): oxidation, addition, and substitution? He queried correctly whether the reaction with I_2 might be more selective than reaction with Br_2, whether an oxidation might occur first and be followed by a substitution or an

addition, and whether the final result was actually a mixture of oxidation and substitution products, from which only (brominated) products were isolated by *Thudichum* and *Maly* (*197*):

> Ueberdies sind uns beide Forscher den einwandsfreien Beweis schuldig geblieben, ob nicht bei der Einwirkung von Brom auf Bilirubin unter den angegebenen Bedingungen neben dem Substitutionsprodukt auch ein Oxydationsprodukt parallel verläuft und ferner, ob nicht zuerst eine Oxydation erfolgt und erst bei weiterer Einwirkung eine Substitution stattfindet, so dass die von den Verfassern erhaltenen Körper einestheils Mischungen von Oxydations- und Substitutionsprodukten waren, andererseits als bromirte Derivate von Oxydationsproduckten des Bilirubins angesehen werden könnten. Man muss sich wundern, dass auf Grund solcher noch ziemlich lückenhafter Arbeiten die Einwirkung der Halogene auf Bilirubin als ein abgeschlossenes Gebiet angesehen wird … 162

He took offense at what he described as an unfounded and prejudiced criticism regarding his work, with no attempt having been made to repeat the reputed experiments, asserted that a comparison between the reactions of Br_2 and I_2 is not generally permissible, especially when I_2 is used at high dilution, and cited *Kekulé* as having already shown that (*197*):

> Die vornehmliche Stütze für seine Behauptungen, dass bei Einwirkung von Jod auf Bilirubin eine Jodsubstitutionsprodukt entstehe, bildet der Analogieschluss, dass, weil Brom auf Bilirubin substituirend wirkt, dies auch zweifellos beim Jod der Fall sein müsse. Ist einsolcher Schluss gerade bei Jod und Brom schon im Allgemeinen nicht statthaft, so ist hier noch zu berücksichtigen, dass Jod in gelöster Form bei seiner Einwirkung auf gelöste organische Substanzen überhaupt nicht substituirend wirkt, zumal in einer so ausserordentlichen Verdünnung, worauf ja schon Kekulé zuerst ausführlich hingewiesen hat[1)]… 163

[1)] Ann. Chem. *131*, 122.

Jolles completed his work (*197*) with the publication of an experimental procedure for the oxidation of bilirubin to biliverdin by I_2, which he characterized by the equation $C_{16}H_{18}N_2O_3 + 2\ I + H_2O = C_{16}H_{18}N_2O_4 + 2\ HI$, and he provided an elemental combustion analysis of the latter as well as a list of the usual characteristic properties: solubility, fluorescence with added $ZnCl_2$, and various color changes upon treatment with acids, including a positive *Gmelin* test (*197*).

It may be noted in the combustion analysis that the %C, H, and N found do not match up well with the theoretical values for the $C_{16}H_{18}N_2O_4$ formula (*197*):

> 1. 0,1846 Grm. Substanz, bei 100° getrocknet, lieferten 0,3928 Grm. CO_2 und 0,0963 Grm. H_2O.
> 2. 0,1708 Grm. Substanz, bei 100° getrocknet, lieferten 14,4 Ccm. Stickstoff bei 728 Mm. und 20° .

Berechnet für Biliverdin $C_{16}H_{18}N_2O_4$:		Gefunden:
C	63,58 %	62,76 %
H	5,96 „	6,27 „
N	9,26 „	8,44 „

164

Yet the total collection of data was apparently sufficient to have convinced *Jolles* that he had in fact prepared biliverdin from bilirubin by the action of I_2 (*197*):

> Aus den vorstehend angeführten Resultaten geht somit mit Sicherheit die Thatsache hervor, dass das durch Einwirkung der alkoholischen Jodlösung auf Bilirubin unter den angegebenen Versuchsbedingungen entstehende Produkt weder ein Jodsubstitutions-, noch ein Jodadditionsprodukt, sondern nur ein Oxydationsproduct darstellt, und zwar ist dasselbe mit Rücksicht auf die Ergebnisse der Elementaranalyse, sowie der charakteristischen Eigenschaften des Körpers als Biliverdin anzusprechen. 165

Jolles' reply was apparently not the *mea culpa* that *Thudichum* had sought. In 1900, a year before his death and beset by *Jolles*' studies as well as others on bile pigment issues that he believed to have put to rest decades earlier, including whether urobilin is present in normal urine, investigators who appeared to repeat or rely upon the errors of previously published work (while neglecting his corrections of such errors), an exasperated *Thudichum* coined the word *Conjecturalchemie* (*191*). He assailed the current crop of bile pigment researchers for having read the published literature only selectively, for not being able to distinguish between conjecture and fact when citing it, and for being out of touch with chemistry. Waxing philosophical *Thudichum* attributed such errors and deficiencies to a continuous deterioration (of scientific knowledge and understanding) ever since the so-called physiological chemistry had suffered separation from overall chemistry in every civilized country as promoted by academic chairs and literary organs (*191*): "Seitdem die sog. physiologische Chemie von der allgemeinen durch Professuren und litterarische Organe abgetrennt worden ist, hat sie in allen Culturländern eine unblässige Verschlechterung erlitten."

Unwilling to let *Jolles* have the last word on the subject of halogen-promoted oxidation of bilirubin to biliverdin, in 1900 *Thudichum* (*191*) wrote (on the subject of the treatment of bilirubin with I_2) that *Jolles*' 57-page article in 1894 (*196*) was completely refuted in his paper published in 1896 (*187*). Yet, in 1899 *Jolles* persisted (*197*), according to *Thudichum*, in attempting to revive a few of his earlier assertions by changing his position, necessitating changes that might befuddle a reader who does not follow the subject (*191*):

> Dadurch wurde eine 57 Seiten lange Abhandlung . . . von Dr. Adolf Jolles in Wien vollständig widerlegt. Nichtsdestoweniger hat derselbe in diesem Journal . . . einige seiner früheren Behauptungen aufzufrischen versucht, zu diesem Zwecke aber seinen Standpunkt so zu verändern sich genöthigt gesehen, dass die Leser, welche dem Gegenstand nicht folgen, darüber orientirt werden sollten. 166

Thudichum continued to object to *Jolles*' published work, accusing him of first using the false oxidation of bilirubin by Br_2 as an analog of the purported oxidation by I_2 – then quietly abandoning it, along with all other false statements based on formulas, results, and processes reported by *Maly* and *Rödeler*. Of course, he strongly objected first to *Jolles*' use of *Maly*'s doubled formula ($C_{32}H_{36}N_4O_6$) for bilirubin – which he said had been determined by no one except to be written on paper – and then to *Jolles*' switching to the *Städeler-Maly* formula ($C_{16}H_{18}N_2O_3$) that, *Thudichum* said, was not defined by any analysis. He complained that *Jolles* had not analyzed his bilirubin, nor had he investigated the biliverdin prepared from

it by *Zuntze*'s method (but not by I_2) but referred only to a *Maly* preparation (which *Thudichum* said did not exist). One gets a better sense of *Thudichum*'s dismay and strong feelings here and later in the original German (*191*):

> Die Basis, auf welche er seine durch gar nicht vorhandene Analogie ihm eingegebene Arbeit zu gründen glaubte, nämlich die schon lange als nicht existirend nachgewiesene Oxydation des Bilirubins durch Brom, ist ihm jetzt unter den Händen entschlüpft. Alle die falschen Angaben, welche er über angebliche Formeln, Resultate und Processe von Rödeler und Maly gemacht hatte, sind ebenfalls aus dem neuen Text weggelassen. Also z. B. anstatt $C_{32}H_{36}N_4O_6$, der von Niemand ermittelten, sondern nur auf dem Papier gemachten, an sich in jeder Beziehung falschen Formel für Bilirubin, giebt er jetzt die ebenfalls ganz falsche, durch keine Analyse oder Verbindung gestützte Formel $C_{16}H_{18}N_2O_3$. Er hat nun nicht etwa Bilirubin analysirt oder durch Verbindungen definirt, oder das daraus durch Zuntz's Methode (aber nicht durch Jod) darstellbare Biliverdin untersucht, sondern spricht von einer angeblichen Methode Maly's, Biliverdin herzustellen, die gar nicht existirt. 167

Unrelentingly, *Thudichum* scoffed at *Jolles*' spectrum analyses of his pigments, complained that the bilirubin spectrum was due as much to impurities in the commercial (allegedly pure) bilirubin as to the pigment itself – saying that all that the spectroscopy proved was the impurity of all of *Jolles*' preparations, without his realizing it. And he broadened his assault to say that it then followed entirely irrefutably that *Jolles*' errors in print (should anyone believe them) would bring forth only confusion and that his quantitative estimates (of bilirubin, using I_2) were falsely called "determinations" and possessed no value whatsoever (*191*):

> Ich hatte nachgewiesen, dass die von Dr. Jolles dem Bilirubin und Biliverdin zugesprochene und von ihm auf einer Tafel mit anderen ähnlichen Neuigkeiten abgebildeten Spectra diesen Körpern nicht zukommen. Er giebt nun jetzt an, dass seine Spectra mit „Substanz" erhalten worden seien, welche er als rein von einem Fabrikanten gekauft habe, während sie doch in der That unrein gewesen sei. Die von ihm skizzirten Absorptionsspectra waren daher Produkte der Unreinigkeiten in seinen Präparaten, und keineswegs der zu erforschenden Substanz selbst. Auf Seite 3 seiner Schrift in Pflüger's Archiv behauptet er die Identität seines aus Galle oder vermeintlichem Bilirubin durch Jod erhaltenen grünen Produkts mit „reinem Biliverdin spectroskopisch" bewiesen zu haben. Alles was er, ohne es zu wissen, bewiesen hat, war die Unreinheit aller seiner Präparate. Daraus folgt nun ganz unwiderleglich, dass er mit seinen Irrthümern, wenn ihnen Jemand traute, nur Wirrwarr hervorbringen würde, jedenfalls aber, dass seine angeblichen Mengeschätzungen, fälschlich Bestimmungen genannt, keinerlei Werth besitzen. 168

And in a final admonishment to *Jolles*, *Thudichum* objected to the former's having written or copied from a statement by *Maly* that no analyses were run on the brominated product from reaction of bilirubin with Br_2. *Thudichum* was emphatic in stating that he had in fact concluded complete elemental combustion analyses on two preparations of dibromo-bilirubin and had long ago refuted *Maly*'s false statement on the subject. His parting words on the polemic with *Jolles*: I herewith protest against the carelessness with which *Jolles* treats the literature (*191*):

> In seinem Aufsatz über diesen Gegenstand in den Wiener Monatsheften, der im Wesentlichen eine Wiederholung des Aufsatzes in diesem Journal ist, sagt Hr. Jolles, ich hätte mein Bromsubstitutionsprodukt des Bilirubins nicht analysirt. Diese Angabe is nicht etwa eine Ermittlung des Hrn. Jolles selbst, sondern sie ist aus einem Aufsatz des weil. Prof. Maly abgeschrieben. Sie is völlig unbegründet. Die Theorie der Bildung des

> Dibrombilirubins ist nicht nur durch die Zunahme des Bilirubins an Brom und das Weggehen des Bromwasserstoffs, sondern auch durch vollständige Elementaranalyse von zwei Präparaten, von denen eines über 20 Grm. wog, bewiesen worden. Die falsche Angabe von Maly habe ich schon lange widerlegt, und ich erhebe hiermit nochmals Protest gegen die Nachlässigkeit, mit welcher Hr. Jolles die Litteratur behandelt. 169

Leaving for the moment his polemic with *Jolles*, *Thudichum* then moved on to address recent work of others on the urinary pigments by firmly reminding us of his own, also in the context of conjectural chemistry, of which he gave many examples from the chemistry of urine, bile, brain, and other organs and essential parts of the body, chiefly in articles in this journal (*Journal für praktische Chemie*) and in more than 30 articles published in English medical journals. Referring to his three most recent publications on the subject (*188–190*), two of them (*188*, *189*) addressed mainly the errors that he had refuted some 25 years earlier, including the purported identity of urobilin (isolated from urine but previously not analyzed) with *Maly*'s hydrobilirubin [one of a mixture of products obtained by treatment of bilirubin with Na(Hg)], he turned his ire again toward *Maly* for having published falsely on the subject and thus having provided the means for physiological chemists who later picked up on the work to incorporate and propagate errors. Though he expressed hope that *Hopkins* and *Garrod* in London (*194*, *195*) would provide a further final rejection of *Maly*'s work, he also chided them for not having recognized that their elemental analysis of urobilin proved it to be nothing more than that discovered by him in 1864, where he had described it as *Omicholin* (*191*), analyzed from eight preparations. Satisfyingly, their urobilin analyzed for 4.11% N and *Thudichum*'s *Omnicholin* for 4.18% N, in contrast to *Maly*'s hydrobilirubin, 9.75% N. Accordingly, no further explanation was required (*191*):

> Ich habe dies an vielen Beispielen aus der Chemie des Harns, der Galle, des Gehirns und anderer Organe und Bestandtheile des Körpers bewiesen, hauptsächlich in Artikeln in diesem Journal, und in mehr als dreissig Artikeln in englischen medicinischen Zeitschriften. Um nicht mit Wiederholungen zu belästigen, weise ich an dieser Stelle auf drei von mir gemachte neueste Mittheilungen hin, welche in Virchow's Archiv für pathol. Anat. un Physiol. und für klin. Med. **150** (1897) 586, daselbst **153** (1898) 154 und **156** (1899) 284 erschienen sind. Zwei derselben betreffen hauptsächlich den schon vor 25 Jahren von mir widerlegten Irrthum, dass das sogenannte Urobilin, eine aus dem Harn isolirte, bisher nicht analysirte Substanz, mit dem Hydrobilirubin, einer aus Bilirubin durch Natriumamalgam erhaltenen Mischung von Pigmenten identisch sei. Diese von Maly in die Welt gesetzte falsche Angabe ist später von den physiologischen Chemikern weiter geschleppt worden, bis sie durch die Untersuchung der HHrn. F. G. Hopkins und A. E. Garrod in London eine weitere, und wie zu hoffen steht, endliche Abweisung erhielt. Letztere haben durch Elementaranalyse bewiesen, dass das Urobilin weiter nichts ist als das von mir im Jahre 1864 entdeckte und genau beschriebene, an acht Präparaten analysirte Omicholin. . . . Ihr Urobilin enthält 4,11% Stickstoff; mein Omicholin 4,18% Stickstoff; Hydrobilirubin dagegen 9,75% Stickstoff. Darnach bedürfen die übrigen Unterschiede keiner weiteren Darlegung. 170

Seemingly unrelentingly, *Thudichum* again took issue with *Jolles*, who nevertheless, by conjectural chemistry and from five centigrams of impure material and mathematical equations added a new pigment (*Bilixanthin*) to the scene. Which *Thudichum* claimed was identical to the *Uroxanthin* obtained from urine that *Jolles* passed off as a new discovery. *Uroxanthin*, the particularly colored material that is differentiated from the characteristic yellow urinary pigment (urochrome) (*191*)

was of course discovered by an earlier professor of physiological chemistry, *Heller*.[58] *Jolles* apparently indicated that unlike *Uromelanin* (*196*) *Uroxanthin* contains *Indigoblau* (indigo blue) as the diagnostic radical. *Thudichum* expressed that the radical was falsely identified with an indigo plant extract and named *Indican*, that the indigo-containing substance of urine, *Heller*'s *Indigogen* or *Uroxanthin*, yielded no sugar and no glucoside and therefore was not identical with indican, the glucoside of the indigo plant and thus there was no justification for use of the word *Indican* in urology. He admonished *Jolles* for having usurped *Heller*'s name, *Uroxanthin*, to apply to a different product, and thereby for violating ethics (*191*):

> Eben weil nun Heller den Namen Uroxanthin für ein jedenfalls genügend gekennzeichnetes Educt gewählt, und sich dieser Name in der Litteratur eingebürgert hat, halte ich seine Anwendung auf ein anderes Produkt nach den Gesetzen der litterarischen Ethik für unerlaubt. 171

Thudichum's final article on bile pigments continued in the same vein, objecting to physiological chemists' penchant for misrepresenting compounds previously discovered and cited *Heller*'s *Urohidin* as an example. He subsequently identified *Heller*'s *Urohidin* as indigo-red or *Indirubin*, because it was colored red and obtained in addition to indigo-blue. Apparently, they had not read *Thudichum*'s 1877 article on *Urohidin*, which could not be an indigo-blue isomer because it analyzed for no nitrogen and contained 80% carbon (*191*):

> Als weiteren Beweis für die nachlässige Art, mit welcher manche physiologische Artikelschreiber mit den besten Entdeckungen der Vorgänger umgehen, erwähne ich das Schicksal von Heller's Urohodin, ein von ihm zuerst hervorgebrachtes Produkt. Dasselbe wurde von Conjecturchemikern, weil es neben dem Indigoblau erhalten wurde, und roth von Farbe war, für Indigoroth oder Indirubin erklärt. Allein die Elementaranalyse machte auch dieser Vermuthung ein Ende. Mein „Experiment über das Urohodin" in Pflüger's Archiv **15** (1877) 346 bewies, dass dasselbe, von einem ungefärbten Urohodinogen durch starke Salzsäure erhalten, keineswegs dem Indigoblau isomer sein kann, da es keinen Stickstoff, wohl aber 80% Kohlenstoff enthält. Seine Aetherlösung zeigte ein specifisches Absorptionsspectrum, in welchem allen Grün durch ein dunkles Band ausgelöscht, ist, wenn Roth und Blau durchscheinen. 172

Thudichum did not spare the new, young investigator *William Küster* from criticism on the crystal morphology and purity of his crystallized bilirubin (*198*, *199*) and the 0.7–1.3% and 1.53–2.89% higher than expected %C and %N values, respectively, found by *Küster*'s elemental combustion analysis – values similar to those from his own macroscopic crystalline bilirubin (*191*):

> Er hat dann ein ganzes Capitel der Beschreibung der Darstellung von angeblich krystallisirtem Bilirubin verfasst, ohne dass dabei auch nur ein einziger Krystall zum Vorschein

[58] *Johann Florian Heller* was born on May 4, 1813 in Iglau, Austria and died on November 21, 1871 in Vienna. He was one of the founders of clinical chemistry and a distinguished pathological chemist who established a laboratory of pathological chemistry at the Wiener Allgemeines Krankenhaus ("AKH" = Vienna's General Hospital). He had studied chemistry in Prague and in Giessen (with *Liebig* and *Wöhler*), researched the chemistry of urine in Vienna, and developed the (well-known) *Heller*'s ring test for albumin in urine. A prize in his name is awarded by the Austrian Association for Clinical Chemistry (ÖGKC).

> gekommen wäre. … Ich lehne daher die von dem Aussehen der Produkte des Hrn. Küster abgeleiteten Schlüsse für Reinheit seiner Produkte ab, und behaupte, dass alle Produkte von Bilirubin aus seinen Processen unrein waren. Den Beweis hat er selbst geführt durch seine Elementaranalysen von Proben, deren Kohlenstoff von 0,7% bis 1,3% zu hoch ist; aber namentlich der Stickstoff ist von 1,53%-2,89% zu hoch, wenn controllirt durch die Verbindungen des nach meiner Methode dargestellten und makroskopisch krystallisirten Bilirubins. 173

He scolded *Küster* for having used *N*,*N*-dimethylaniline as a bilirubin crystallization solvent. He deemed it entirely unsuitable because it is a base, can react easily with other compounds at its high boiling temperature, and remains attached to the precipitate. *Thudichum* stated that *Küster*'s crystals so obtained contained some of the base. He was apparently dismayed that *Küster*'s yield of crystallized product was only one-third of the starting bilirubin, took issue with *Kuster*'s attempted oxidation to biliverdin using PbO_2 (which *Thudichum* claimed had been shown long ago to fail). And after progressing to what he called "erroneous reports on biological-chemical matter in periodical journals of chemistry and medicine", for which he provided examples, he could not end the discourse without providing a reason for having chastised *Maly*, who apparently had the temerity to attack *Thudichum*'s research on urine. *Thudichum* was then immediately forced to convince *Maly* in public that his relevant reports were incorrect from beginning to end and would consequently draw no belief whatsoever from informed readers (*191*):

> Zuletzt muss ich die Leser warnen vor einigen Ausfällen, welche Professor Maly in seinem Jahresbericht als Zeugniss der Fortsetzung seiner Behandlung der Wahrheit gegen meine Forschungen über das Hirn gemacht hat, die er vorher durch ein Plagiat bewiesen hatte, so dass ich ihn öffentlich zu überführen geradezu gezwungen war. Die betreffenden „Berichte" des Hrn. Maly sind unrichtig von Anfang bis zu Ende und haben daher bei unterrichteten Lesern keinerlei Glauben gefunden. 174

Thudichum was the consummate scientist of his era, an extraordinarily talented and meticulous researcher. An apparent deep thinker and broadly interested in medicine, disease, and chemical physiology. Like the author's former colleague at UCLA, the renowned *Nobel* Prize candidate and physical organic chemist *Saul Winstein* (1912–1969), he showed no mercy when confronted with what he considered to be suspect or shoddy work and especially its continued promulgation. Yet *Winstein* was more often correct in polemic discourse than was *Thudichum*. *Thudichum* clearly and forcefully expressed his beliefs, backed by experiment and the scientific logic of the age, when assailing contemporary and (especially) new investigators of bile pigments, as well as those investigators and their theories of the research area for which he is most famous: the chemistry of the brain (*163*, *200*):

> It is surprising to find how little the chemical relations of the brain are understood by physiologists, and chemists of profession. They ignore the broadest facts, and maintain the most absurd fallacy which has ever disfigured animal chemistry, namely, the so-called doctrine of protagon. They thereby impede the progress of science, and confuse the minds of those who are desirous to learn and to work. …

2.13 Knowledge of Bilirubin Near the End of the 19th Century

By the close of the 19th century, the then greatest names associated with bilirubin had passed from the scene: *Thenard* (1777–1857), who carried out early isolations of bilirubin and biliverdin from bile and discovered a goldmine of the yellow pigment in the bile duct of a deceased elephant; *Berzelius* (1777–1848), who labored for nearly 40 years to isolate and purify bilirubin (*Cholepyrrhin*, *Gallenbraun*), biliverdin (*Gallengrün*), and bilifulvin from bile – and for very different reasons dominated the field of chemistry; *Tiedemann* (1781–1861) and *Gmelin* (1788–1853), who showed that air (oxygen) was required to convert the yellow pigment to the green and discovered the characteristic display of colors from treatment with HNO_3 that became the enduring and famous *Gmelin* reaction (or diagnostic color test) for bilirubin in bile, urine, *etc.*; *Scherer* (1814–1869), who isolated a green pigment from bile and jaundiced urine and carried out one of the earliest elemental combustion analyses; *Heintz* (1817–1880), who created an improved separation method for isolating bilirubin, carried out elemental combustion analyses of it and wrote formulas for bilirubin as $C_{31}H_{18}N_2O_9$ as better than $C_{32}H_{18}N_2O_9$, and for biliverdin as $C_{16}H_9NO_5$ or its double formula, $C_{32}H_{18}N_2O_{10}$ to fit the data; *Valentiner*, who while working in *Friedrich Theodor von Frerichs*' lab in Göttingen in the 1840s introduced $CHCl_3$ extraction to isolate bilirubin and showed that it was probably identical to *Virchow*'s hematoidin cited in 1847; *Brücke* (1819–1892), who improved *Valentiner*'s isolation method to obtain the purest bilirubin to date, as well as a collection of related pigments and analyzed "ash-free" samples by combustion; *Städeler* (1821–1871), who isolated "purified" bilirubin and biliverdin and conducted C, H, N elemental combustion analyses that corresponded first to the formula $C_{18}H_9NO_4$, then later to $C_{32}H_{18}N_2O_6$ for bilirubin, and $C_{32}H_{20}N_2O_{10}$ for biliverdin; *Maly* (1839–1891), who also conducted C, H elemental combustion analyses of isolated bilirubin and suggested the formula $C_{16}H_{18}N_2O_3$ for it, while the %C, H, N of his biliverdin was fit to $C_{16}H_{20}N_2O_5$; and *Thudichum* (1829–1901), who carried out detailed isolations and combustion analyses to show that bilirubin had the (empirical) formula $C_9H_9NO_2$, biliverdin had the formula $C_8H_9NO_2$, and who became a dominating voice on bile pigments in the last half of the 19th century.

With the completion of the important new bile pigment research of the mid-late 1800s by *Städeler*, *Maly*, and *Thudichum*, understanding bilirubin had reached its final stage before the advent of the era of chemical degradation and synthesis. Certainly, by the late 1870s "animal" or organic chemistry had progressed to the use of a wide range of chemicals, reagents, and solvents that illustrated a rapidly maturing chemical science. New and improved methods for isolating bilirubin from gallstones and bile had been developed. Many elemental combustion analyses had been run, albeit few of them on apparently homogeneous samples, from which conflicting molecular or empirical formulas were extracted. An elementary form of absorption spectroscopy in the visible region had been introduced and was used for comparing pigments. Yet despite the many advances in knowledge of bilirubin and biliverdin, and the discovery of a probable relationship between the pigments of

blood and bile, a correct molecular formula was still debatable, a molecular weight had been determined only from the material balance in a chemical reaction devoid of knowledge of almost any aspect of chemical structure, and the purity or homogeneity of bilirubin, while vastly improved over that in the early part of the 19th century, was still suspect.

Polemics, however satisfying, disillusioning, or disenabling, failed to produce new knowledge on the structure of bilirubin itself. Thus, near the close of the 19th century, the status of the knowledge of bilirubin could be summarized briefly by *Arthur Gamgee* in 1893 (*201*):

Bilirubin $C_{32}H_{36}N_4O_8$

(Synonyms: Cholepyrrhin, Biliphæïn, Bilifulvin, Hæmatoidin[6]*).*

Occurrence. Bilirubin occurs in the yellow or reddish-yellow bile of man and carnivorous animals, in the bile of the pig and occasionally in the bile of the herbivora which have been long without food. It also occurs in the contents of the small intestine and is a normal constituent of the blood serum of the horse … It is further a common constituent of gall-stones; it occurs in the urine, and stains the conjuctivae and skin, in cases of jaundice. In old blood extravasations it occurs in microscopic crystals which were first discovered by Virchow and by him called hæmatoidin. …

Physical and Chemical Characters.

Colour and crystalline form. Bilirubin occurs in an amorphous and in a crystalline condition. In the former it presents the appearance of an orange-coloured powder resembling sulphide of antimony; in the latter it has the colour of crystallized chromic acid. Examined under the microscope, crystalline bilirubin exhibits orange-coloured rhombic tables, in which the obtuse angles are often rounded off. When crystallising from solutions which are not quite pure (containing cholesterin, &c.) better formed crystals are obtained than is the case when the solutions contain no such impurities (Hoppe-Seyler …).

Solubility. Bilirubin is insoluble in water, almost insoluble in ether and very sparingly soluble in alcohol. It is readily soluble in chloroform especially with heat; it is likewise soluble (though to a much less extent than in chloroform) in benzol, carbon disulphide, amyl alcohol, and glycerin. These fluids dissolve enough however to acquire a yellow or a brown red colour. Solutions of bilirubin which contain 1 part in 500000 exhibit a perceptible yellow colour when a layer 1·5 cm. thick is observed (Hoppe-Seyler).

Bilirubin is readily soluble in dilute solutions of sodium and potassium hydrate and ammonia, and if the solutions be kept from contact with air or with oxygen, it can be reprecipitated from them by addition of hydrochloric acid.

It is important to notice that solutions of bilirubin in alkalies do not yield the colouring matter to chloroform. A chloroformic solution of the colouring matter shaken with dilute sodium or potassium hydrate is at once decolourised; on the other hand a similar alkaline solution

[6] The name hæmatoidin is only applied to bilirubin when occurring in old extravasations of blood.

of bilirubin if acidulated and shaken with chloroform at once gives up its colouring matter, which is dissolved by the chloroform and imparts to it a much less brownish-yellow colour.

Bilirubin forms compounds with bases of which several have been studied. The Na-compound is obtained by precipitating a dark orange solution of bilirubin in sodium hydrate by means of a concentrated solution of caustic soda.

The Ca-compound is obtained by precipitating an ammoniacal solution of bilirubin with calcium chloride. The precipitate is rust-coloured, flocculent, and insoluble in water, alcohol, ether and chloroform. It has the composition indicated by the formula $C_{32}H_{34}N_4O_6$. Ca. When this compound is dried *in vacuo* over sulphuric acid it is of a dark-green colour with a metalic lustre, but when powdered it has a dark-brown colour.

By the action of barium chloride, lead acetate, and nitrate of silver on ammoniacal solutions of bilirubin, compounds similar to the calcium compound can be obtained. The silver compound occurs in violet-coloured flakes and is not reduced even when the liquid in which it is suspended is boiled. Bilirubin, as Maly observes, shews by the compounds which it forms, that it has the characters of a weak acid.

Composition and formula. Heintz[1] was the first chemist to make an ultimate analysis of bilirubin, and assigned to it the formula $C_{16}H_{18}N_2O_5$. The method which he followed in the preparation of the substance, which was not until later obtained crystallised, renders it certain that it was not free from impurities, and the results of his analysis may therefore be left out of consideration. The same objection does not apply to Städeler's methods. The results of his work have been absolutely confirmed by the more recent and exhaustive researches of Maly, as well as by Hoppe-Seyler[2].

Both Städeler and Maly from their analyses deduced for bilirubin the formula $C_{16}H_{18}N_2O_3$. Thudichum[3], on the other hand, has assigned to bilirubin the formula $C_9H_9NO_2$, which neither agrees with the concordant analytical results of Städeler and Maly, nor fits in with many facts with which we are acquainted. The reader will see at a glance how considerable are the differences in the percentage of the various elements calculated from Städeler and Maly's formula on the one hand, and from that of Thudichum on the other.

	(Städeler and Maly.) $C_{16}H_{18}N_2O_3$ or $C_{32}H_{36}N_4O_6$	(Thudichum.) $C_9H_9NO_2$
Carbon	67·13	66·25
Hydrogen	9·79	5·52
Nitrogen	9·79	8·59
Oxygen	16·79	19·64
	100·00	100·00

[1] Heintz, Poggendorff's *Annalen*, Vol. LXXXIV, p. 106.

[2] "Ausser diesen Ergebnissen der Untersuchungen von Städeler sind noch von Maly und von Thudichum solche veröffentlicht, von denen die Resultate Maly's Bestätigung der Untersuchungen Städeler's geben. Die Analysen von Hoppe-Seyler lassen gleichfalls keinen Zweifel an der Richtigkeit der Formel von Städeler und von Maly." Hoppe-Seyler, *Handbuch d. Phys. u. Path. Chem. Analys*., 6th ed. (1893), p. 226.

[3] Thudichum, *Journ. f. prakt. Chem*., Vol. CIV. (1868), p. 193.

Quite apart from the remarkable concordance of the results of Städeler and of Maly, an examination of all facts bearing on the question[1] has led chemists to the opinion that the formula of Städeler and Maly, or probably a multiple of it, is correct. The various reactions are best explained by doubling Städeler's formula.

Action of nitric acid on bilirubin 'Gmelin's reaction'.

When bilirubin is treated with pure dilute nitric acid (containing 20 per cent. of HNO_3) no change occurs at ordinary temperatures. When the solution is heated, however, dark-violet resinous flakes are formed which as the temperature rises assume a light-brown colour and ultimately dissolve, yielding a yellow-coloured liquid.

Pure concentrated nitric acid acts in the cold and a cherry-red liquid is obtained which retains its colour for many days. Nitric acid which has a slightly yellow colour and which contains nitrous acid[2] (as the nitric acid of commerce does) gives rise in solutions which contain bilirubin, to a remarkable play of colours already referred to as 'Gmelin's reaction.' The reaction may be tried with a dilute alkaline solution of bilirubin, with diluted bile, or with any liquid, such as the urine of jaundice, which contains bilirubin.

Various methods of exhibiting Gmelin's reaction may be adopted. The most common is to pour some of the solution to be tested into a test tube containing nitric acid, so that the two liquids are not mixed. Near the line of junction the colour-reaction at once commences to develope [sic], and a succession of zones of colour appear, the tints being, from above downwards, as follows: –green, blue, violet, red and reddish-yellow. These tints represent the successive stages of the reaction, the first being green and the last the reddish-yellow, which is observed in the region where the oxidising action is most intense, viz. in close proximity to the nitric acid.

Instead of employing a test tube, a few drops of diluted bile, or bilious urine may be poured upon a flat plate, so that a thin layer of liquid is obtained. On now adding a drop or two of coloured nitric acid, wherever the acid falls a series of concentric coloured rings of beautiful is developed, the succession of tints being the same as in the experiment previously described.

The delicacy of 'Gmelin's reaction' is such that it permits of the detection of bilirubin in solutions which contain only 1 part of the colouring matter in from seventy- to eighty-thousand parts of water. It must be remembered that in order to be sure of the presence of bilirubin the whole series of tints must be observed, as *lutein* (yellow crystalline matter obtained from corpora lutea, from the yolk of egg, and which is also present in the liquor sanguinis of some animals), when treated with nitric acid, exhibits a green and also a blue tint very similar to those developed in Gmelin's reaction. The spectroscopic characters of lutein are, however, sufficiently distinctive to enable the observer to ascertain whether this substance is present in a solution or not.

Each tint in Gmelin's reaction corresponds apparently to a definite chemical change, probably to a definite oxidation product. The green tint is due to the production of biliverdin, which as will be afterwards shewn is the first stage in the oxidation of bilirubin. The blue tint is due to an imperfectly studied body termed bilicyanin; the final reddish-orange colour is due to choletelin.

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[1] Such as the results of the analysis of the calcium compound of bilirubin, of Maly's tribromobilirubin, no less than the relation of bilirubin to biliverdin; to the latter point reference will again be made.

[2] If the acid is too highly coloured (i.e. if the amount of nitrous acid and of nitrogen peroxide be large) it exerts so energetic an action on the bilirubin that the successive stages of Gmelin's reaction cannot be properly observed.

Though *Thudichum* might have become upset with *Gamgee*'s assertion that the *Städeler-Maly* formula for bilirubin was superior to his own and that *Städeler*'s doubled formula best explains the various reactions of the pigment, his major research projects at that time were focused on the chemistry of the brain. However, if he could not be bothered with *Gamgee*, he found the energy to rebut the new researchers of the 1890s who had probably innocently stepped too hard on the wrong set of toes. Yet, while the tirades continued, newer important studies on bilirubin, regarding its molecular weight, its elemental combustion analysis, and its degradation into identifiable small fragments were underway by a new set of investigators: *Nencki*, *Teeple*, and *Küster*.

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