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 alchemy antecedents and stimulated by a cascade of discoveries from many notables, including the seminal contributions of the French nobleman scientist Lavoisier1 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 CO2 and H2O 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

1Antoine-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.
principle and seminal experiment became the basis for the modern process of combustion analysis introduced some 50 years later by Liebig.  

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 à 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 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 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 N₂, 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, Cadet⁵ 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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⁵Louis-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.

¹Please 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 K₂CO₃, 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 Vallisneri⁶ 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, Haller⁷ 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 Salle⁸, 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 gall-bladder 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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⁶Antonio Vallisneri, 1661–1730, a professor of practical medicine in Padua.
⁷Albertus (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.
⁸François 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\textsuperscript{9}, who reported them in his \textit{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\textsuperscript{10}, Fourcroy\textsuperscript{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 spermaceti (37, 42, 43). The name for the white crystals would later (1815) be given as cholestérine (Greek: χόλη for bile; στερεά for solid) by Chevreul\textsuperscript{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.

\textsuperscript{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 \textit{Dictionnaire de Chymie} first published in 1766, with subsequent expanded editions that followed.

\textsuperscript{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.

\textsuperscript{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 Médecine 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.

\textsuperscript{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 Académie 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…

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, abit…

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\(^\text{13}\) and Gmelin\(^\text{14}\) 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 abgibt. Alle Arten von Galle, sowohl von Säugthieren, als Vögeln, als Amphibien und Fischen, die wir in dieser Beziehung untersuchten, färbten sich bei allmählichen Zufügen von Salpetersäure

\(^{13}\) 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.

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…

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, H2SO4, and acetic acid, only nitric acid (and the traces of NO2 present) is required for the spectrum of colors. The work established what became famous as the Gmelin 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… – 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 Rosenbach15 (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 CHCl₃, treated with one drop of fuming HNO₃, 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.
   - 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 alkalis.
   - 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 CO₂ 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, working with Thenard, who upgraded the combustion process by admixing KClO₃ 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 KClO₃, 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 CaCl₂ and CO₂ 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 CO₂ and H₂O 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.

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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 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 overthrew ancient dogma (Vitalism) by creating an organic substance (urea) from its elements by heating an inorganic source (NH₄CNO, ammonium

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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, 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 millennia, 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 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 H2SO4, HNO3, or HCl, in all cases a yellow material was formed, along with little resin. Using, variously, alcohol, ether, BaCl2, lead acetate, from the precipitated barium or lead salts and the supernatant he obtained

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19 Jöns Jakob Berzelius was born on August 20, 1779 in Västersund 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 bittersweet taste). As Thomson wrote (30): “The name picromel is, I presume, from \( \pi\kappa\rho\omicron\zeta \): bitter, and \( \mu\epsilon\lambda\tau: \) 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:

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

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 acetic 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 alkalis, alkaline earths, and alkaline acetates decompose and dissolve it: the former by depriving it of its combined acid; the latter, by furnishing it with acetic 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 redens 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 barytes 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 redens 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.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>907.4</td>
</tr>
<tr>
<td>Biliary matter</td>
<td>80.0</td>
</tr>
<tr>
<td>Mucus of the gall-bladder, dissolved in the bile</td>
<td>3.0</td>
</tr>
<tr>
<td>Alkalies and salts (common to all secreted fluids)</td>
<td>9.6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1000.00</strong></td>
</tr>
</tbody>
</table>

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$_2$SO$_4$ (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, an esteemed physician and porphyrinologist who studied under Hans Fischer in 1931–1932 (62):

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*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 HNO₃, 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 editor. 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 Gallensteinen 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 unaufloslichen Schleimrückstand bleiben, wovon aber gerade das Gegenteil 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 übrigen Bestandthiele der Galle besitzen noch weniger Farbe, und können deshalb noch weniger als das färbende Princip derselben betrachtet werden.


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 oxydirt.en Zustand des Farbestoffs derselben.

Gmelin also noted that gallstones contain the pigment of bile and give the same color test (Gmelin reaction) with HNO₃. 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 stärkere Färbung an, und gab eine Flüssigkeit, die anfangs gelb war, sich jedoch an der Luft grasgrün und mit Salpetersäure blassroth färbe und durch Chlor entfärbt wurde.


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.

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):


22Marc-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 Gallenstein, 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 auffässlich. 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 bekomen 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 gleich 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. —

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):


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).
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):


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 $C_4H_{14}N_2O_6$, thought then to be a di-salt with ammonia. Taurine is still known today as a major component of bile, but with the formula $C_2H_3NO_3S (\overset{\sim}{O_3SCH_2CH_2NH_4^+})$. 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 $C_4H_7NO_3S$, or $C_2H_3NO_3S$.

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, Fromhertz, 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/mal-odorous 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 NH₃ in dry distillation – doubtless it was not pure cholic acid, and clearly both carbon-hydrogen and nitrogen combustion analysis were being 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) Na₂CO₃ and (NH₄)₂CO₃; (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):

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 aufflüsslich wird. Legt man noch die zwischen Gallenstoff und Süssholzzucker bestehende Ähnlichkeit 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; dies 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 hieraus 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.

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 (NH₄OH) dissolved the yellow pigment. The latter changed color to grass-green in air and became pale red with HNO₃ and lost its color with Cl₂. 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 HNO₃ 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 HNO₃ 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 H₂SO₄ restored the first color, etc. (68):
2.5 Bilirubin and Biliverdin Separation from Bile by the Middle 19th Century

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 (NH₄OH) 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 O₂ from air. When strongly saturated with HNO₃, 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 HNO₃, 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 HNO₃, 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):


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 H₂SO₄, 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 H₂SO₄. First ox-bile was evaporated over H₂SO₄ 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 alcohol 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 BaCl₂ in H₂O 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):


The residual ethanolic solutions from approach (1) that contained free BaO/ Ba(OH)₂ was precipitated as BaCO₃ with CO₂ 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 (πικροζ, bitter, and μελιτ, honey). Isolated as the metamorphosis products of Bilin were (as named by Berzelius): Fellinsäure (from Fel fellis, bile), Acidum fellicum, Cholinsäure (from χολη, bile), Acidum cholonicium, and Dyslysin (from δυζ, difficult, and λνδιζ, 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):


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.


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 BaCl₂. Two different barium precipitates were obtained, the first from addition of BaCl₂, 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,
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 BaCl₂ – a method similar to (1) but still employing BaCl₂ 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):


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):


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 H₂S, 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 Krystalle an, um welche sich dann bei fortgesetzter Verdunstung ein braunrothes Extract bildet. Diese Krystalle sind es, die ich Bilifulvin genannt habe.

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 *Jahresbericht*, 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
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 HNO₃, 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):


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 brandgelben Krankheitsprodukte aus der Galle verschiedener Thiere durch Alkali unter dem Einfluss der Luft hervorgebracht wird.

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*:

während der Analyse metamorphosirt wird, ist. Dieser Farbstoff verdient einen eigenthüm-lichen Namen, man kann ihn *Cholepyrrhin* (von χολή, Galle, und πυρρός, brandgelb) nennen.

*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):

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*, *Platter*, *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\textsuperscript{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 \textit{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 \textit{aq. potash} and \textit{NH}_4\text{OH} (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 \textit{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):

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\]

\textit{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 \textit{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, \textit{Platner} finished with the comment that the pigment obtained by \textit{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):

\[
\begin{quote}
Schliesslich will ich noch einige Bemerkungen über den Farbstoff der Galle machen.
Die Galle wird bekanntlich durch Säuren nach und nach grün, wenn zugleich die Luft
\end{quote}
\]

\textsuperscript{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 \textit{Liebig} at Giessen and became lecturer, then Professor at the University of Christiana in Norway in 1851, and Professor at Tübingen following \textit{Gmelin}’s death. He moved to Würzburg in 1870.
Simon\textsuperscript{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: 
\textit{Biliphäin}, which Berzelius had named \textit{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

\textit{a.} The most important colouring matter of the bile is that to which it owes its characteristic brownish yellow tint. It is termed \textit{cholepyrrhin} by Berzelius, and \textit{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. \textit{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 \textit{biliverdin} by Berzelius. It is no

\textsuperscript{24}Johann Franz Simon (1807–1848) received the Dr. phil. in 1838 in Berlin, habilitated as \textit{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* 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 HNO₃ (92):

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25Johann 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.

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 CO₂ and H₂O, 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.

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<tr>
<td>%C</td>
<td>67.409</td>
<td>67.761</td>
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<td>%H</td>
<td>7.692</td>
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<td>%N</td>
<td>6.704</td>
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<td>%O</td>
<td>18.195</td>
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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 who wrote a famous series of monographs (Archiv für pathologische Anatomie und

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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 Übergänge 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 Ausführungswege 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 gleicherzässige, diffuse; sehr bald sammelt sich aber der Farbstoff in kleine, unlösliche, bräunliche 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 HNO₃ 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 HNO₃ 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 HNO₃ 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 dies in den meisten Fällen ganz richtig. Allein nicht in allen Fällen bewirkt die

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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 Privadozent 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 Physikalische 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 eintritte.

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.

bei allmählichen 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 Farbe auf, und scheint dadurch vollkommen 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 allmählichen Oxydation beruhen. Clorogas wirkt auf dieses Pigment gleich der Salpetersäure, nur etwas schneller; grössere Mengen von Chlor bleichen den Farbstoff vollkommen und schlagen weisse Flocken nieder.


Aus diesem Grunde sind die Angaben über die Eigenschaften dieser Stoffe so verschieden; man vergleiche Berzelius, Scherer, Heintz, Platner 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.


Darstellung. Früher empfahl man gewöhnlich zur Darstellung des Gallenfarbstoffes, 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 ganz richtig angegeben hat und jeder vorurtheilsfreie Beobachter sich leicht überzeugen kann), selbst in solchen Concrementen, die grösstentheils aus Cholesterin bestehen.

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 filtrierten 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.


Physiologisches Verhalten.


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 Biliphæin (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 Biliphæin 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 biliphein, 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. Städelier 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 CHCl₃.

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:

<table>
<thead>
<tr>
<th></th>
<th>% I</th>
<th>% II</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>73.237</td>
<td>73.212</td>
</tr>
<tr>
<td>H</td>
<td>6.306</td>
<td>6.313</td>
</tr>
<tr>
<td>N</td>
<td>14.434</td>
<td>14.434</td>
</tr>
<tr>
<td>O</td>
<td>6.023</td>
<td>6.041</td>
</tr>
</tbody>
</table>

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 NH₃ 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

<table>
<thead>
<tr>
<th></th>
<th>Hein-1</th>
<th>Hein-2A*</th>
<th>Hein-2B**</th>
<th>Scherer-1A</th>
<th>Scherer-1B</th>
<th>Scherer-2†</th>
<th>Hein-3A</th>
<th>Hein-3B</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>69.68</td>
<td>67.96</td>
<td>68.13</td>
<td>73.237</td>
<td>73.212</td>
<td>62.491</td>
<td>58.26</td>
<td>58.5</td>
</tr>
<tr>
<td>O</td>
<td>13.88</td>
<td>15.89</td>
<td>15.49</td>
<td>6.023</td>
<td>6.041</td>
<td>23.122</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ash</td>
<td>9.326</td>
<td>4.301</td>
<td>4.33</td>
<td>41.79</td>
<td>41.79</td>
<td>†</td>
<td>9.326</td>
<td></td>
</tr>
</tbody>
</table>

* bei der Verbrennung im Platschiffchen (from combustion in a Pt boat)
** bei der Verbrennung zwischen Kuperoxyl (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 Na₂CO₃ followed by precipitation with HCl, with or without heating, treatment with alcohol, precipitation with basified acetic acid-lead oxide, then BaCl₂ induced precipitation followed by treatment with H₂SO₄, 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

<table>
<thead>
<tr>
<th></th>
<th>%C</th>
<th>%H</th>
<th>%N</th>
<th>%O</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>65.5</td>
<td>67.409</td>
<td>67.761</td>
<td>61.837</td>
<td>74</td>
</tr>
<tr>
<td>N</td>
<td>6.704</td>
<td>6.704</td>
<td>7.074</td>
<td>9.080</td>
<td>14.4</td>
</tr>
<tr>
<td>O</td>
<td>18.195</td>
<td>17.987</td>
<td>17.246</td>
<td>24.246</td>
<td>23.192</td>
</tr>
<tr>
<td>Ash</td>
<td>7.833</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 CaCO₃, along with some MgCO₃. 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 Galtenbraun 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 H₂-blanketed flask, crude Gallenbraun, previously washed (agitation) by hydrochloric acid and water, was dissolved in aqueous Na₂CO₃ 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 CO₂ 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 Wasserstoffgasentwicklung begonnen, und nachdem so viel dieses Gases entwickelt worden war, dass man annehmen konnte, auch in der im Kolben befindlichen kohlensauren Natronlösung befände 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 durch 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 herausergossen, 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 CaCl₂ and a barium salt with BaCl₂. 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 HNO₃. Treatment of a dilute alkaline solution of Biliphäin with excess HNO₃ containing some aqueous HNO₂ 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\textsubscript{31}H\textsubscript{18}N\textsubscript{2}O\textsubscript{9} (formula wt 562) molecular formula was a better fit than C\textsubscript{32}H\textsubscript{18}N\textsubscript{2}O\textsubscript{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)

<table>
<thead>
<tr>
<th>%I</th>
<th>II</th>
<th>III*</th>
<th>IV*</th>
<th>C\textsubscript{32}H\textsubscript{18}N\textsubscript{2}O\textsubscript{9}</th>
<th>C\textsubscript{33}H\textsubscript{18}N\textsubscript{2}O\textsubscript{9}</th>
<th>V</th>
<th>C\textsubscript{16}H\textsubscript{9}NO\textsubscript{5}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>60.70</td>
<td>60.71</td>
<td>61.06</td>
<td>61.03</td>
<td>61.94</td>
<td>61.18</td>
<td>60.04</td>
</tr>
<tr>
<td>H</td>
<td>6.05</td>
<td>6.02</td>
<td>6.09</td>
<td>6.06</td>
<td>5.80</td>
<td>5.92</td>
<td>5.84</td>
</tr>
<tr>
<td>O</td>
<td>23.73</td>
<td>23.79</td>
<td>23.23</td>
<td>23.69</td>
<td>25.59</td>
<td>25.16</td>
<td>25.16</td>
</tr>
<tr>
<td>Ash</td>
<td>0.29</td>
<td>0.37</td>
<td>0.045</td>
<td>0.079</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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\textsubscript{16}H\textsubscript{9}NO\textsubscript{5} (formula wt 295), although C\textsubscript{32}H\textsubscript{18}N\textsubscript{2}O\textsubscript{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 Biliveredins 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 vermuten. 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, trieft 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 Fettt oder Cholesterin. Die Abweichung der Resultate seiner Analyse (er hat zudem nur eine Kohlenstoff- und Wasserstoffbestimmung ausgeführt) ist demnach erklärlich. Es wäre zu wünschen, dass andere Chemiker, denen
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 $\text{Ag}^+$, $\text{Ba}^{+2}$, $\text{Ca}^{+2}$, 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, Valentiner28 (107), while working in von Frerichs’ laboratory in Breslau...
and in Berlin, discovered that CHCl₃ digestion of pulverized gallstones, which had been exhaustively washed with ethanol and with ether to remove cholesterol, fats, and other solubles, produced a yellow CHCl₃ solution. Upon evaporation of the CHCl₃, 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 tierische 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 Gruppierung. Um grössere Krystalle rein zu erhalten, war es vortheilhaft, der Chloroformlösung vor dem Verdunsten etwas tierisches 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.

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. H₂SO₄ 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):


In yet another experiment using CHCl₃ extraction, human and animal bile shaken with CHCl₃ 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 CHCl₃ extracted hematoidin and left behind dark green bile. In his experiments Valentiner thus discovered that the yellow-red pigment was soluble in CHCl₃ but the green was not, a distinction in relative solubility shared by bilirubin and biliverdin.

Very soon after Valentiner’s report, in 1859, Brücke sought to answer a question that he posed as to whether small amounts of bile pigment could still be detected after successful extraction using CHCl₃, *i.e.* how completely does CHCl₃ remove the pigment, with detection to be monitored using the very sensitive Gmelin color change reaction from HNO₃ (109):

In contrast to Valentiner, Brücke focused on extracting bile rather than gallstones. He found that CHCl₃ extracted a yellow pigment from human gallbladders. After separation by decantation, the CHCl₃ 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.

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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 CHCl₃ extraction and evaporated it to dryness. At this point one might wonder how complete the separation of bile from CHCl₃ was and whether CHCl₃ (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 CHCl₃-extracted bile, pulverized it, and digested the powder with CHCl₃, decanted the CHCl₃ and then added fresh CHCl₃ to the residue along with as much water as needed to dissolve the bile. The aqueous bile was then repeatedly extracted with fresh CHCl₃ and the color of the CHCl₃ 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):


Though Brücke found his results at odds with Valentiner’s description, a finding which begged explanation, he determined that the CHCl₃-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

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 CHCl₃ 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 CHCl₃ 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 CHCl₃ extracted Biliphäin from bile, it did not extract biliverdin. That is, Biliphäin was soluble in CHCl₃ but biliverdin was not. Brücke also learned or knew that while Biliphäin was insoluble in alcohol, biliverdin was soluble. Thus, not only did the CHCl₃ 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):


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 CHCl₃ 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 CHCl₃ 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 CHCl₃ to afford again a yellow solution from which crystals were able to be obtained upon distilling off the CHCl₃. 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 Na₂CO₃ 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 CHCl₃. Thus, the conclusions to be drawn from the work of Valentiner and Brücke were: (i) CHCl₃ 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) CHCl₃ extraction leaves biliverdin behind; (iii) Biliphäin is soluble in CHCl₃; biliverdin is insoluble; (iv) biliverdin is soluble in ethanol; Biliphäin is insoluble; (v) pure Biliphäin can be separated from biliverdin by a CHCl₃ wash; and (vi) pure biliverdin can be isolated by extraction into ethanol following air oxidation of pure Biliphäin.

2.8 Hämatoïdin, 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 Hämatoïdin (hematoidin) (94):

In Beziehung auf die Gefässe will ich diejenigen Fälle angeben, wo man am sichersten auf die Anwesenheit von Hämatoidin-Krystallen rechnen darf.
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):

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 kristallinisich 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 H₂SO₄ + HNO₃, Virchow then concluded that “Eine Vergleichung unserer Pigmente mit dem 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


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 speziell in einer ausgedehnten Zerstörung von Blutkörperchen 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. . . . *Hematoïdin* 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):


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:

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<th>I</th>
<th>II</th>
<th>III</th>
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<tr>
<td>Kohlenstoff</td>
<td>65,0460</td>
<td>65,8510</td>
<td>–</td>
</tr>
<tr>
<td>Wasserstoff</td>
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<td>6,4650</td>
<td>–</td>
</tr>
<tr>
<td>Stickstoff</td>
<td>–</td>
<td>–</td>
<td>10,5050</td>
</tr>
<tr>
<td>Sauerstoff</td>
<td>18,0888</td>
<td>17,1788</td>
<td>–</td>
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<tr>
<td>Asche</td>
<td>0,0002</td>
<td>0,0002</td>
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Hämatozin besteht im Mittel von 5 Analysen aus:

\[
C_{44}H_{22}N_3O_6Fe
\]

oder in 100 Theilen aus:

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<tr>
<td>Kohlenstoff</td>
<td>65,84</td>
</tr>
<tr>
<td>Wasserstoff</td>
<td>5,37</td>
</tr>
<tr>
<td>Stickstoff</td>
<td>10,40</td>
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<tr>
<td>Sauerstoff</td>
<td>11,75</td>
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<td>Eisen</td>
<td>6,64</td>
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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.

By the late 1850s, Valentiner (107), the first to discover that CHCl₃ extracted nearly pure pigment from gallstones and bile, would find the brown-red crystalline residue from evaporation of the CHCl₃ 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 CHCl₃ evaporation or whether it had been precipitated by acidification of an aqueous Na₂CO₃ 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
Hämatoïdin contained Biliphäin (or vice versa) – and thus that bile pigments originated from the pigment of red cells, hematin.

Apparently, Frerichs\textsuperscript{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 denselben identischen Farbstoff zurückführten. \textsuperscript{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

\textsuperscript{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).


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 Gallensäuren wird durch die Pettenkofer’sche Probe vergebens gesucht. Nur in einem Falle, wo eine ungewöhnliche 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 injizirt 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 haematoïdine. From this it appears possible, nay probable, that, as in extravasations, haematoïdine 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. 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 poured 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 dyspnea, as, for instance, in one dog, which died from edema 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\(_2\)SO\(_4\) 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\(_2\)SO\(_4\) solution was kept cold or heated. When sodium glycocholate was treated with conc. H\(_2\)SO\(_4\) 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\(_2\)CO\(_3\). Upon treatment with HNO\(_3\), the color changes observed (green\(\rightarrow\)reddish\(\rightarrow\)yellow) were reminiscent of a slower reacting positive Gmelin test for bilirubin or biliverdin. From colorless sodium taurocholate treated with conc. H\(_2\)SO\(_4\) 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\(_2\)SO\(_4\), and there is no reason to believe that the pigmented material obtained from chemical transformation of a bile acid in H\(_2\)SO\(_4\) 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\textsuperscript{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.\textsuperscript{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):

\textsuperscript{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$_2$SO$_4$ to produce a purple coloration.

\textsuperscript{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 Hoppé’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 hematin 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 Städelerr 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 CHCl₃ as an extraction solvent to remove

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34 Georg Andreas Karl Stüdele 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):


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. Valentiner [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. Valentiner 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 Valentins’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 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

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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):

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 *Valentinier* 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 *Städelers* 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. H₂SO₄ 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 Königl. 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 früheren 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 pigmentiertem 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 näher 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.
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$_2$SO$_4$ 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$_2$O produced flakes, which when gently heated turned violet, then blue after a few seconds. Similarly, filter paper coated with the aqueous H$_2$SO$_4$ 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 saffrongelber, 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 prachtvolle gefärbbte 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 blau 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.

To determine whether bile acids might become bile pigments, Frerichs and Städelers treated glycocholic acid with H$_2$SO$_4$ 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$_2$SO$_4$ 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$_2$O. 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$_2$SO$_4$ solution for six hours produced substantially the same results, except the blue residue produced by addition of H$_2$O 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 HNO₃, 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 HNO₃. 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 (I29):

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 (°H₃NCH₂CH₂SO₃⁻) is decomposed into glycine (°H₃NCH₂CO₂⁻) and *Saligenin* in the liver (I29):

Für jetzt beschränken wir uns darauf, auf die Ähnlichkeit der natürlichen Gallenpigmente mit den von uns erhaltenen Zersetzungsprodukten 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 Zersetzungspunkt der Gallensäuren. Wir sprachen schon früher . . . die Ansicht aus, dass dieser Farbstoff als Nebenprodukt 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 H₂O, 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 HNO₃ produced the most beautiful display of color changes characteristic of bile pigments. And the Pettenkofer reaction was negative (129):


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 gestetzt 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$_2$SO$_4$, 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$_2$SO$_4$ 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 stärkerer Verdünnung hat man die zu prüfende Flüssigkeit 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$_2$SO$_4$ to a small sample of urine, plus a trace of sugar, and warming to evaporation in a porcelain dish to display the colors (130): Geling 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$_2$O and HOSO$_3$ for H$_2$SO$_4$, 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$_2$O 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$_2$SO$_4$) 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$_2$SO$_4$ (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 unaugst 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 modifizirtem Verfahren wenigstens Spuren von Gallensäuren unzweideutig nachweisbar waren.

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 H₂SO₄ and did not change upon addition of sugar solution. Crude concentrated HNO₃ 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 H₂SO₄ and HNO₃. 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 HNO₃ produced a barely perceptible Gmelin reaction. Addition of conc. H₂SO₄ 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 HNO₃ produced a distinct Gmelin reaction; added conc. H₂SO₄ 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. H₂SO₄ 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 H₂SO₄ 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.

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 HNO₃. Added conc. H₂SO₄ 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 H₂SO₄ 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 Hilfe 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.


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):

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):


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 injicierte, 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; häufi ge Wiederholung der Versuche und vorurtheilsfreie Interpretation der erlangten Resultate wird uns allmählich 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 hämatogenous 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 enigmatic 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 hematin 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, Jaffe37 (132, 133), too, thought that hematoidin and Bilifulvin were identical, following his experiments on old brain hemorrhages, which he dried and extracted with CHCl₃, 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, \textit{Hoppe-Seyler} (134) isolated the same pigment, with the same properties from a cyst in the breast. \textit{Städelier} (135) criticized the crystal morphology cited by \textit{Valentiner} (107, 108), finding the crystal angles of the orange-colored elliptic, \textit{i.e.} crystals (of bilirubin) isolated following evaporation of CHCl$_3$, as being very different from those of hematoidin, which never had convex surfaces. \textit{Holm}, working in Zürich with \textit{Städelier} (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, \textit{Salkowski} (137) found somewhat different results from \textit{Holm}: similar crystal forms, solubility, and positive \textit{Gmelin} test. In contrast, \textit{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, \textit{Thudichum} (1829–1901, see below, Section 2.9.2) claimed that the others had examined not hematoidin but \textit{Lutein} (lutein, also xanthophyll) – and that lutein differed altogether from bilirubin. At essentially the same time, \textit{Kühne} (123–125) and \textit{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 \textit{Manual of General Pathology} (English translation published in 1876) \textit{Ernst Wagner} wrote that blood extravasations consisted of not just hematoidin but also contain bilirubin (and probably other compounds). Following \textit{Thudichum}, \textit{Wagner} preferred to call the pigment lutein rather than hematoidin. He cited the studies of \textit{Holm} and \textit{Städelier} (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.

\textit{Holm} and \textit{Städelier} (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 alkalis, and is soluble in alkalis, the former not; therefore from a solution of the latter in chloroform it may be separated by agitation with caustic alkalis,
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); haematoxidin, 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 haematoxidin from a strumous cyst had all the peculiarities of bilirubin. He concludes that the haematoxidin (from corpora lutea) investigated by Holm was not pure, or that there are different kinds of haematoxidin.

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 haematoxidin and bilirubin were not identical, but in 1883, Hermann (141) identified haematoxidin 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 haematoxidin 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, haemochromogen. 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 haemoglobin, large quantities of water, chloroform, and ether, common salt solution, glycerine, toluylendiamine, arseniurretted 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 haemoglobinuria 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 haematoxidin 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 haematoxidin 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 hämatoidin.” 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 haemoglobin.

… Haematoporphyrin, on partial reduction with hydriodic acid, yields mesoporphyrin:

\[
\text{C}_{33}\text{H}_{38}\text{O}_{6}\text{N}_{4} + 2\text{H}_{2} = \text{C}_{33}\text{H}_{38}\text{O}_{4}\text{N}_{4} + 2\text{H}_{2}\text{O}
\]

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

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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, H₂O, and HỌ. 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 C₂H₆O (Berzelius), C₂H₁₀O, H₂O (Liebig), or C₃H₇₈, H₄O₂ (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 CHCl₃ 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 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 CHCl₃ 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 HNO₃, 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 C₁₄H₉NO₃, did not correspond in any way, a re-analysis of hematoidin yielded a satisfactory correspondence to C₃₀H₁₈N₂O₆, as comes from the following composition (129):

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 Universitāt Göttingen as Privadozent 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).
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):

\[
2 \text{C}_{18}\text{H}_{11}\text{NO}_6 + 2 \text{O} \rightarrow \text{C}_2\text{O}_4 + \text{C}_4\text{H}_4\text{O}_4 + \text{C}_{30}\text{H}_{18}\text{N}_2\text{O}_6
\]

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 \( \text{C}_{15}\text{H}_6\text{NO}_4 \) is calculated.

\[
\begin{array}{lcccc}
\text{Berechnet}^1 & \text{Gefunden} \\
\hline
\text{30 Aeq. Kohlenstoff [C]} & 180 & 65,69 & 65,85 & 65,05 \\
\text{18 Wasserstoff [H]} & 18 & 6,57 & 6,47 & 6,37 \\
\text{2 Stickstoff [N]} & 28 & 10,22 & 10,50 & 10,50 \\
\text{6 Sauerstoff [O]} & 48 & 17,52 & 17,18 & 18,08 \\
\hline
\text{274} & 100,00 & 100,00 & 100,00 \\
\end{array}
\]

\( \text{Berechnet}^1 = \text{Calculated}; \text{Gefunden} = \text{Found}. \) 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 \( \text{C}_{15}\text{H}_6\text{N}_2\text{O}_4 \).
93 2.9 Bile Pigment Isolation, Purification, and Combustion Analysis…

<table>
<thead>
<tr>
<th>Elements</th>
<th>Calculated formula</th>
<th>Actual Result of Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 equivalents of carbon</td>
<td>108</td>
<td>66.26</td>
</tr>
<tr>
<td>9 &quot; &quot; hydrogen</td>
<td>9</td>
<td>5.52</td>
</tr>
<tr>
<td>1 &quot; &quot; nitrogen</td>
<td>14</td>
<td>8.59</td>
</tr>
<tr>
<td>4 &quot; &quot; oxygen</td>
<td>32</td>
<td>19.63</td>
</tr>
<tr>
<td></td>
<td>163</td>
<td>100.00</td>
</tr>
</tbody>
</table>

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

\[
\text{C}_{16}\text{H}_9\text{NO}_4 + \text{C}_2\text{H}_3 \\text{H} = \text{C}_{18}\text{H}_9\text{NO}_4
\]

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 \( \text{C}_{18}\text{H}_9\text{NO}_4 \), which is somewhat different from his analytical data that predicted \( \text{C}_{30}\text{H}_{18}\text{N}_2\text{O}_6 \) for hematoidin (151), and also that from Robin (115, 116), thus Städeler wrote (151): \( \text{C}_{14}\text{H}_9\text{NO}_3 \).

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 \( \text{C}_{18}\text{H}_{19}\text{N}_2\text{O}_4 \) for Cholepyrrhin and \( \text{C}_{15}\text{H}_{17}\text{N}_2\text{O}_3 \) 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 CHCl₃ 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: C₃₀H₁₈N₂O₆ mit der Formel des Biliverdins: C₁₆H₉NO₅ oder C₃₂H₁₈N₂O₁₀, 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.

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 converted 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.

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 CHCl₃ 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 CHCl₃ extraction, was treated with dilute HCl to dissolve a large quantity of calcium and magnesium salts and evolve CO₂. The resulting dark brown residue, after washing and drying, yielded a large amount of pigment into boiling CHCl₃, thus suggesting to Städeler that the majority of the pigment had been originally bound up as salts. Evaporation of this CHCl₃ 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 CHCl₃ extraction above, contained a considerable amount of Gallenroth (bilirubin), albeit in impure condition. After as much “brown pigment” as possible had been extracted with CHCl₃, 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 CHCl₃. The residue, after all of the washings/ extractions, was insoluble in H₂O, alcohol, ether, CHCl₃, 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 CHCl₃-extracted Gallenroth, taking it through several cycles of dissolving it in CHCl₃, 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 C₁₈H₉NO₄ 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 CHCl₃ 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 filtrierte Lösung verdunstet und der Rückstand mit Aether und Weingeist gewaschen. Der abfiltrierte Weingeist zeigt sich immer mehr oder minder grün bis grünlichbraun gefärbt, während das Bilirubin als ein lebhaft rothes bis orangerothes, körnig-kristallinisches Pulver zurückblieb.

Bei der Analyse des so gereinigten Farbstoffes wurden Zahlen erhalten, die mit keiner annehmbaren Formel genügend übereinstimmen, worauf 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. H₂SO₄ 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 H₂S. (It is unclear how bilirubin, which contains no sulfur, might evolve H₂S 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 KNO₃) followed by acidification of the residue with HCl produced no turbidity upon addition of BaCl₂ (135):


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 C₁₅H₁₈N₂O₆.[41]

<table>
<thead>
<tr>
<th>berechnet</th>
<th></th>
<th>I.</th>
<th>II.</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 Aeq Kohlenstoff</td>
<td>192</td>
<td>67,13</td>
<td>67,15</td>
</tr>
<tr>
<td>18 „ Wasserstoff</td>
<td>18</td>
<td>6,29</td>
<td>6,27</td>
</tr>
<tr>
<td>2 „ Stickstoff</td>
<td>28</td>
<td>9,79</td>
<td>9,59</td>
</tr>
<tr>
<td>6 „ Sauerstoff</td>
<td>48</td>
<td>16,79</td>
<td>16,99</td>
</tr>
<tr>
<td>286</td>
<td>100,00</td>
<td>100,00</td>
<td>–</td>
</tr>
</tbody>
</table>

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 Sb₂S₃); 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 C₁₆H₁₉N₂O₅.
in ethanol but dissolved in cold CHCl₃ to give a yellow to yellow-orange solution. The more crystalline it was, the more difficult it was to effect dissolution in CHCl₃; continuous heating was required. And it was noted that pure CHCl₃ became rapidly acidic and generated phosgene. In such CHCl₃ the erstwhile yellow color turned to green. But when the CHCl₃ contained a bit of ethanol, there was no color change. (And so commercial CHCl₃ typically contains a little ethanol as stabilizer). Benzene and CS₂ 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 (NH₄OH) 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 K₂CrO₄. At a factor of $5 \times 10^4$, 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.

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 direkten Sonnenlicht, während sie sich im zerstreuten Licht nur langsam zersetzen. Sie werden allmählich 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.

Städeler noted some differences between solutions of bilirubin in aqueous ammonia, NaOH, and Na₂CO₃, and that these aqueous basic solutions extract all of the pigment from its solution in CHCl₃. He indicated that compounds of bilirubin
with “earths” and heavy metal oxides were insoluble or barely soluble in $\text{H}_2\text{O}$. A voluminous rust-colored calcium compound was precipitated by addition of $\text{CaCl}_2$ 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 $\text{CHCl}_3$, and when heated in the last two solvents gave only a weakly yellow color. In a similar way, the salts with $\text{BaCl}_2$, sugar of lead, $\text{Pb(OAc)}_2$, and $\text{AgNO}_3$ 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 $\text{C}_{32}\text{H}_{17}\text{N}_2\text{O}_6\text{Ca}$:

\[0.2549 \text{ grm. hinterliessen beim Verbrennen, Anfeuchten der Asche mit kohlensaurem Ammoniak und Trocknen bei } 130^\circ \text{ 0,0414 grm. kohlensauren Kalk, übereinstimmend mit der Formel:}\]

\[\text{C}_{32}\text{H}_{17}\text{CaN}_2\text{O}_6. \text{ Die Rechnung verlangt 9,18 pC. Kalk; gefunden wurden 9,10 pC.} \]

Städel er treated bilirubin systematically with $\text{HNO}_3$, producing new results and a calibration of the Gmelin reaction. Warming bilirubin with dilute $\text{HNO}_3$ ($20\% \text{ H}_2\text{O}$) 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 $\text{HNO}_3$ ($30\% \text{ H}_2\text{O}$) 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 $\text{HNO}_3$ 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):


If bilirubin was dissolved in commercial conc. $\text{HNO}_3$, 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 $\text{HNO}_3$ and mix them with an approximately equal volume of alcohol. Upon addition of $\text{HNO}_3$, 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 cm³ 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 Säure zusetzt, so erhält man die bekannte

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 HNO₃ by neutralizing with ammonia. All this produced at first a green flocculent precipitate that gradually became blue. After washing the precipitate with H₂O, 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 CHCl₃ solution of bilirubin by mixing in 1–2 drops of HNO₃ 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):


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 die violette Farbenentwicklung 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 Weingeizzusatz.

Städeler noted that bilirubin dissolved in cold, conc. H₂SO₄ to produce a brownish liquid that gradually turned violet-green. Addition of H₂O separated dark green, nearly black flakes that dissolved in alcohol with a marvellous violet color. Addition of HNO₃ 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 (Städelert 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. Städelert was not able to investigate the resulting compound further, which he believed to remain probably in a similar relationship to bilirubin 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 bilirubin formula given by Städelert 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.

Before turning from the pigments of gallstones to the pigments of human bile, Städelert 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 CHCl₃. 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. Städelert was convinced that the green pigment was the same as that which Heintz had analyzed by combustion and for which he established the formulas \( C_{16}H_{9}NO_5 \), or \( C_{32}H_{18}N_2O_{10} \) \((95)\). These formulas would be produced from Städelert’s bilirubin formula by the addition of four oxygen atoms. Yet from his own analyses, Städelert remained doubtful \((135)\):


Dieses grüne Pigment ist ohne allen Zweifel das von Heintz . . . analysirte Biliverdin, wofür er die Formel \( C_{16}H_{9}NO_5 \) 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:

\[
\text{C}_{32}\text{H}_{16}\text{N}_{2}\text{O}_{6} + 4\ O = \text{C}_{32}\text{H}_{18}\text{N}_{2}\text{O}_{10}
\]

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 \(\text{C}_{32}\text{H}_{20}\text{N}_{2}\text{O}_{10}\) than to Heintz’s \(\text{C}_{32}\text{H}_{18}\text{N}_{2}\text{O}_{10}\) formula (97), while the hydrogen content found lay in the middle between the two formulas (135):

\[
\begin{array}{c|c|c|c}
\text{Kohlenstoff} & 60,00 & 60,04 & 60,38 \\
\text{Wasserstoff} & 6,25 & 5,84 & 5,66 \\
\text{Stickstoff} & 8,75 & 8,53 & 8,80 \\
\text{Sauerstoff} & 25,00 & 25,59 & 25,16 \\
\hline
100,00 & 100,00 & 100,00.
\end{array}
\]

Wahrscheinlich was das von Heintz analysierte 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.

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. \(\text{Na}_2\text{CO}_3\) 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):

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

$^{[1]}$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):

$$2 C_{32}H_{18}N_2O_6 + 4 \text{HO} = C_{22}H_{20}N_2O_6 + C_{32}H_{20}N_2O_{10}$$

$\text{Bilirubin} \quad \text{Biliverdin}$

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

$$C_{32}H_{20}N_2O_{10} + 2 \text{HO} = C_{32}H_{22}N_2O_{12}$$

$\text{Bilirubin} \quad \text{Biliprasin}$


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$_2$O.
So dargestellt bildet das Bilifuscin eine fast schwarze glänzende spröde Masse, die beim Zerreiben ein dunkelbraunes, etwas in’s Olivenfarbene ziehendes Pulver gibt. Es ist frei von Aschenbestandtheilen, verhält sich beim Erhitzen eben so wie das Bilirubin und gibt 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; übereinstimmend mit der Formel C32H20N2O8:

<table>
<thead>
<tr>
<th>berechnet</th>
<th>gefunden</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 Aeq.</td>
<td>192</td>
</tr>
<tr>
<td>20 „</td>
<td>20</td>
</tr>
<tr>
<td>2 „</td>
<td>28</td>
</tr>
<tr>
<td>8 „</td>
<td>64</td>
</tr>
<tr>
<td>304</td>
<td>100,00</td>
</tr>
</tbody>
</table>

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:

\[\text{C}_{32}\text{H}_{18}\text{N}_2\text{O}_6 \quad \text{C}_{32}\text{H}_{20}\text{N}_2\text{O}_6\]

Bilirubin Bilifuscin.

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 H₂O, ether, and CHCl₃ (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 NH₃ or NaOH, producing a dark-brown solution from which brown flakes precipitated upon addition of HCl. Mixing an aq. NH₃ solution with CaCl₂ 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 Städelier. It was pulverized, washed with ether and with CHCl₃, 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 C₃₂H₂₂N₂O₁₂, 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 Grm. gaben 0,073 Chlorsilber.
Diese Verhältnisse führen zu Formel C_{32}H_{22}N_{2}O_{12}:

<table>
<thead>
<tr>
<th>berechnet</th>
<th>gefunden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeq. Kohlenstoff</td>
<td>32</td>
</tr>
<tr>
<td>„ Wasserstoff</td>
<td>22</td>
</tr>
<tr>
<td>„ Stickstoff</td>
<td>2</td>
</tr>
<tr>
<td>„ Sauerstoff</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

Städeler summarized the properties of Biliprasin: insoluble in H_{2}O, 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_{2}CO_{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_{2}O, 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 löslich 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 NO₄\(^{[43]}\) 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 HNO₃ containing NO₄ \(\equiv NO_2\), 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):

\[
\begin{align*}
\text{C}_{32}\text{H}_{16}\text{N}_2\text{O}_6 & \quad + \quad 2\text{HO} & \quad = \quad \text{C}_{32}\text{H}_{20}\text{N}_2\text{O}_2 \\
\text{Bilirubin} & \quad + \quad 2\text{HO} + 2\text{O} & \quad = \quad \text{Bilifuscin} \\
\text{C}_{32}\text{H}_{20}\text{N}_2\text{O}_{10} & \quad + \quad 2\text{HO} & \quad = \quad \text{C}_{32}\text{H}_{22}\text{N}_2\text{O}_{12} \\
\text{Biliverdin} & \quad + \quad 2\text{HO} + 2\text{O} & \quad = \quad \text{Biliprasin} \\
\text{Bilihumin} &
\end{align*}
\]

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 dunklen 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):

\[^{43}\text{N.B. NO}_4 = NO_2\] 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 anschliesst, während unreine Chloroformlösungen ganz gewöhnlich krystallinisches Bilirubin liefern. Die krystallinische Ausscheidung scheint bedingt zu sein oder doch sehr befördert zu werden durch die Gegenwart gewisser fremder Stoffe, ebenso wie zur krystallinischen Ausscheidung des Teichmann’schen Hämins aus essigsauerer Lösung die Gegenwart irgend welcher Chlorometalle erforderlich ist. Ich wählte daher die Galle, um das Bilirubin in messbarer Form darzustellen. War der darin vorkommende rothe Farbstoff wirklich identisch mit dem Hämatoidin, wie Valentiner annimmt, so musste er sich bei richtig gewählter Behandlung auch in der so regelmässig auftretenden Hämatoidinform gewinnen lassen. 90

StädeleL had learned from his own experiments that in addition to its solubility in CHCl₃, bilirubin was sufficiently soluble in CS₂ and in benzene to be extracted from gallstones. Thus, he extracted human bile with these three solvents. From repeated experiments using CHCl₃, 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 orangefarbenen elliptischen Blättchen oder sehr kleiner, fast rechtwinkelter Tafeln, deren Winkelverhältnisse sehr wesentlich verschieden sind von denen des Hämatoidins. 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 Hämatoidinform näherte. 91

Using CS₂ 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 CHCl₃, one by CS₂, 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 CHCl₃ 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):

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 Städel er 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):

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, Städel er 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):

<table>
<thead>
<tr>
<th></th>
<th>Bilirubin</th>
<th>Hämatoïdin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kohlenstoff</td>
<td>67,15</td>
<td>67,11</td>
</tr>
<tr>
<td>Wasserstoff</td>
<td>6,27</td>
<td>6,12</td>
</tr>
<tr>
<td>Stickstoff</td>
<td>9,59</td>
<td>10,51</td>
</tr>
<tr>
<td>Sauerstoff</td>
<td>16,99</td>
<td>17,17</td>
</tr>
<tr>
<td><strong>100,00</strong></td>
<td><strong>100,00</strong></td>
<td></td>
</tr>
</tbody>
</table>

*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 C₁₄H₉NO₃ but that the formula C₃₀H₁₈N₂O₆ 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 C₃₀H₁₆N₂O₆; so, it and bilirubin (C₃₂H₁₈N₂O₆) 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 C₁₄H₉NO₃ für das Hämatoidin 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 C₃₀H₁₈N₂O₆ gelangt; nur der Wasserstoff ist in diesem Falle um 1/10 und 2/10 pC. geringer gefunden, als der Formel entspricht. – Dass Bilirubin und Hämatoidin nahe verwandte Körper sind, ergibt sich schon aus der grossen Ähnlichkeit der Formeln. Enthielte das Hämatoidin 2 Aeq. Wasserstoff weniger, hätte es also die Formel C₃₀H₁₆N₂O₆, so würde es mit dem Bilirubin, C₃₂H₁₈N₂O₆, in eine homologe Reihe gehören, und damit wären die mehrfachen Ähnlichkeiten in den Eigenschaften genügend erklärt. Doch darüber kann nur durch neue Analysen entschieden werden.

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 Zersetzungsprodukt, 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.

All these and a third green compound isolated several years earlier by Städeler (when the CHCl₃ 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 C₃₂H₁₈.₅N₂.₅O₁₀ 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. H₂SO₄, a chromogen that precipitated in resin-like flakes upon addition of H₂O. If the H₂SO₄ solution were warmed briefly in the absence of air, the precipitated flakes were colorless or greenish, but after standing 24 hours in conc. H₂SO₄ the solution showed a beautiful dichroism that was orange-colored or brownish with a striking pure green transmitted light. Addition of H₂O 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 “NO₄”-containing
HNO₃ (presumably it was NO₂; NO₄ 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 Städelar) to signify a relationship between the synthetic and natural pigment. With this perspective, Städelar 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):

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 H₂O 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):

A new idea apparently struck Städelar 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):


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 HNO₃. 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.

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 CHCl₃ extractions that the solvent was freed of HCl (from its decomposition to COCl₂ 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 hamstring 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 | Calculated for |
| % | A⁵ | B⁶ | C₁₂H₁₈N₂O₆ | C₁₆H₂₀N₂O₈ | D | E | C₁₆H₁₈N₂O₆ | C₁₄H₂₄N₂O₁₂ |
| C | 67.15 | 67.11 | 66.52 | 67.13 | 66.26 |
| H | 6.27 | 6.12 | 6.00 | 6.29 | 8.52 |
| N | 9.59 | – | 8.70 | 9.79 | 8.59 |
| O | 16.99 | – | 18.78 | 16.79 | 19.63 |
| C | 67.15 | 67.11 | 66.52 | 67.13 | 66.26 | 65.85 | 65.05 | 65.69 | 64.12 |
| H | 6.47 | 6.37 | 6.57 | 6.87 |
| N | 10.50 | 10.50 | 10.22 | 10.69 |
| O | 17.18 | 18.08 | 17.52 | 18.32 |

| Bilifuscina | Biliprasina | Biliverdinb |
| % | Experimental | Calculated for | Experimental | Calculated for | Experimental | Calculated for |
| 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 |

³⁴N.B. Combustion analyses may at times be more important in revealing the presence of impurities than in characterizing the intended compound.

⁴⁴Städeler’s data from reference (135), Heintz’s data from reference (97), Hematoidin experimental data from Robin (115, 116), Städeler’s formula (135) from Robin’s experimental data, Robin’s formula from his experimental data (115, 116)
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-Württemberg.) 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 “cholopheine” (synonymous with Cholepyrrhin, Bilipherain, and Bilifalvin); 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 Bilirubine 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 H₂SO₄, followed by precipitating with or without Ba(OH)₂, (NH₄)₂S, acidifying with HCl, basifying with NH₄OH 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 cholopheine (= Cholepyrrhin = Bilipherain) 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 Städelier (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 cholopheine, 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, cholopheine could be subjected. Four elementary analyses, agreeing with each other, led to the empirical formula C₁₃H₇₆N₂O₉ for cholopheine; but one analysis of cholochloine, the green colouring matter hitherto termed biliverdine, obtained from the brown by oxidation, led Heintz to the formula C₁₆H₆₈N₂O₅. The improbability of the suggestion that cholopheine, in order to pass into cholochloine, should take up only half an equivalent of oxygen, Heintz met by assuming the formula of cholopheine to be C₁₃H₇₆N₂O₉, 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 cholopheine 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 cholopheine 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.
115

The only combustion analyses data directly cited were those of Städeler for purified *Cholepyrrhin*, obtained from gallstones by CHCl₃ extractions, and for which the formula C₁₈H₉NO₄ 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 C₁₈H₉NO₄ was calculated.

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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 CHCl₃ 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 cholopheaine 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 (N₂O) followed by HNO₃, 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 hemato- 
din, but neither type of crystal could be isolated from the surrounding syrup. Various manipulations of cholochromic acid were engaged: nearly insoluble in H₂O; 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 AgNO₃, 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 Städelier (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, CHCl₃ 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 H₂O (the same way a cook mixes flour for dough) then bathed in hot H₂O with vigorous stirring before being allowed to stand for two days. The water was drained and the solid left behind was thoroughly washed with H₂O 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 CO₂ and H₂S. Thudichum found it better to let the HCl do its work on the solid without heating. The solid was washed free of HCl with H₂O 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 CHCl₃. (It may be important to note that Thudichum, like Städeler before him, took precaution with the CHCl₃, which then doubtless lacked the ethanol stabilizer found nowadays in commercial CHCl₃, because it commonly became acidic by reaction with air and light while it partially decomposed into HCl and phosgene.) The CHCl₃ solution was filtered from the solid, and the residue was again heated in fresh CHCl₃ while certain measures were used to avoid losses of CHCl₃. Then with evidence of great care, Thudichum removed the red CHCl₃ solution from the solid by siphoning, presumably to minimize exposure to air, and distilled off most of the CHCl₃. This left a red residue with some green spots admixed, which was washed on a filtration funnel with CHCl₃ until it was red and no longer co-mixed with green, while the CHCl₃ 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 CHCl₃ and precipitating the concentrated solution by adding absolute alcohol.
Repeated CHCl₃ 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 CHCl₃ and further processed to purification.

Prior to the use of CHCl₃ 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 CHCl₃, and Thudichum examined the crystals in considerable detail (107):

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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 CHCl₃ 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):


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 vor- sichtiges Umkrystallisiren konnte die rothe Modification stets theilweise in die braune verwandelt werden. Es ist daher klar, dass das krystallisirte oder krystallinsiche purpur- braune 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 photo-chemistry] 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 *Städelers’s*: insoluble in H₂O 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 CS₂ and in benzene, and had its best solubility in CHCl₃: 1.7 parts per 1,000 parts CHCl₃ to form a beautiful dark red solution, or about the same solubility as seen today with bilirubin. Sunlight (presumably on the CHCl₃ 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):


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$_9$H$_6$NO) for Cholephäin, a name he used interchangeably with bilirubin (152):

\begin{table}[h]
\centering
\begin{tabular}{lccccccc}
  & I. & II. & III. & IV. & V. & VI. & Mittel \\
\hline
C & 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 \\
\hline
\multicolumn{7}{c}{100,00}
\end{tabular}
\caption{Vergleich der Empirie und Theorie der Elementar-Zusammensetzung des Cholephäins.}
\end{table}

Diese Zahlen führen zur Formel $\text{C}_9\text{H}_6\text{NO}_2$\textsuperscript{47}, deren Theorie mit obigen Thatsachen folgendermassen sich vergleicht:

\textsuperscript{46}One might guess that at least one was biliverdin-IX$\alpha$, possibly contaminated with XIII$\alpha$.
\textsuperscript{47}N.B. Thudichum probably meant “Formel $\text{C}_9\text{H}_6\text{NO}_2$.”
Writing an element’s symbol with a bar through it, such as $\bar{C}$ and $\bar{O}$, 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$_9$H$_9$NO$_2$ 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 Umwandlungen 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$_9$H$_8$(NH$_4$)NO$_2$ + H$_2$O] 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 AgNO₃). The reddish-brown precipitate thus obtained was dried under vacuum over H₂SO₄ 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 (C₉H₆NO₂), were found to be consistent with the neutral hydrated formula, C₉H₁₀AgNO₃ (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 C₉H₉NO₂ 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 C₉H₉AgNO₃ erfordert. Wie anom... 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 C₉H₉NO₂ ausgedrückt wird.

Vergleich der Theorie und Empirie des im Vacuo getrockneten Silber-Cholephäinats.

<table>
<thead>
<tr>
<th>Symbole</th>
<th>At.-Gew.</th>
<th>In 100 Th.</th>
<th>a.</th>
<th>b.</th>
<th>c.</th>
<th>Mittel</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₉</td>
<td>108</td>
<td>37,30</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H₁₀</td>
<td>10</td>
<td>3,47</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ag</td>
<td>108</td>
<td>37,50</td>
<td>37,63</td>
<td>37,52</td>
<td>37,03</td>
<td>37,39</td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Θ₁</td>
<td>48</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>288</td>
<td></td>
<td></td>
<td></td>
<td>107</td>
</tr>
</tbody>
</table>

What Thudichum called the basic silver salt was prepared from Cholephäin dissolved in aq. NH₃ and precipitated by addition of AgNO₃ and HNO₃ (152):

Eine kleine Menge Cholephäin, welche wiederholt durch Lös en 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 abgestumpft. Der jetzt erscheinende Niederschlag liess die Flüssigkeit farblos; er wurde mit Wasser gewaschen und in der Leere getrocknet.

Combustion analysis for silver predicted the formula C₉H₇Ag₂NO₂, with two silver atoms replacing two hydrogen atoms in Thudichum’s empirical formula C₉H₉NO₂ (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 Alkalitätsgrad 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 $\mathrm{C}_9\mathrm{H}_7\mathrm{Ag}_2\mathrm{NO}_2$. 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 $\mathrm{C}_9\mathrm{H}_7\mathrm{PbNO}_2$, 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.**

<table>
<thead>
<tr>
<th>Symbole</th>
<th>At.-Gew.</th>
<th>In 100 Th.</th>
<th>a.</th>
<th>b.</th>
<th>c.</th>
<th>Mittel</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mathrm{C}_9$</td>
<td>108</td>
<td>28,69</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$\mathrm{H}_7$</td>
<td>7</td>
<td>1,85</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$\mathrm{Ag}_2$</td>
<td>216</td>
<td>57,29</td>
<td>56,81</td>
<td>56,41</td>
<td>55,86</td>
<td>56,27</td>
</tr>
<tr>
<td>$\mathrm{N}$</td>
<td>14</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$\mathrm{O}_2$</td>
<td>32</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Σ</strong></td>
<td><strong>377</strong></td>
<td><strong>109</strong></td>
<td><strong>–</strong></td>
<td><strong>–</strong></td>
<td><strong>–</strong></td>
<td><strong>–</strong></td>
</tr>
</tbody>
</table>

The barium salt was also prepared from an aqueous solution of *Cholephäin* in excess $\mathrm{NH}_3$ by precipitating with added $\mathrm{BaCl}_2$. The resultant green precipitate, which *Thudichum* designated a neutral barium *Cholephäinat*, was, after drying, combusted. The analysis data, for $\mathrm{C}$, $\mathrm{H}$, and $\mathrm{Ba}$, were determined to be consistent with the formula $\mathrm{C}_{18}\mathrm{H}_{20}\mathrm{BaN}_2\mathrm{O}_6$, or $(\mathrm{C}_9\mathrm{H}_{10}\mathrm{NO}_3)_2\mathrm{Ba}$ (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 der selben zusammenschweißt; außerdem treten zwei Moleküle Wasser in die Verbindung ein.

**Vergleich der Theorie und Empirie des Baryumcholephäinats,**

$\mathrm{C}_{18}\mathrm{H}_{20}\mathrm{BaN}_2\mathrm{O}_6$.

<table>
<thead>
<tr>
<th>Symbole</th>
<th>At.-Gew.</th>
<th>In 100 Th.</th>
<th>a.</th>
<th>b.</th>
<th>c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mathrm{C}_{18}$</td>
<td>216</td>
<td>43,46</td>
<td>–</td>
<td>–</td>
<td>44,58</td>
</tr>
<tr>
<td>$\mathrm{H}_{20}$</td>
<td>20</td>
<td>4,02</td>
<td>–</td>
<td>–</td>
<td>3,98</td>
</tr>
<tr>
<td>$\mathrm{Ba}$</td>
<td>137</td>
<td>27,56</td>
<td>27,56</td>
<td>27,55</td>
<td>–</td>
</tr>
<tr>
<td>$\mathrm{N}_2$</td>
<td>28</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$\mathrm{O}_6$</td>
<td>96</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Σ</strong></td>
<td><strong>497</strong></td>
<td><strong>110</strong></td>
<td><strong>–</strong></td>
<td><strong>–</strong></td>
<td><strong>–</strong></td>
</tr>
</tbody>
</table>

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 $\mathrm{BaCl}_2$ with excess *Cholephäin*. The precipitate was washed with $\mathrm{H}_2\mathrm{O}$, 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 $\mathrm{C}_{27}\mathrm{H}_{39}\mathrm{BaN}_3\mathrm{O}_8$ (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

\[
\begin{align*}
1 & \text{Baryum-Cholephäinat,} & \text{C}_{18}\text{H}_{29}\text{BaN}_{2}\text{O}_{6} & = 497 \text{ At. Gew.} \\
1 & \text{Cholephäin,} & \text{C}_{9}\text{H}_{9}\text{N}_{2}\text{O}_{2} & = 163 \text{ At. Gew.} \\
& \text{so erhalten wir} & \text{C}_{27}\text{H}_{29}\text{BaN}_{3}\text{O}_{8} & = 660 \text{ At. Gew.}
\end{align*}
\]

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

In like manner, the neutral and half-acid calcium Cholephäinats were prepared and analyzed to indicate probable formulas \(\text{C}_{18}\text{H}_{20}\text{CaN}_{2}\text{O}_{6}\) for the former and \(\text{C}_{27}\text{H}_{29}\text{CaN}_{3}\text{O}_{8}\) 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 100º nicht ausgetrieben werden.

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

### Tabelle 1

<table>
<thead>
<tr>
<th>Symbole</th>
<th>At.-Gew.</th>
<th>In 100 Th.</th>
<th>a.</th>
<th>b.</th>
<th>c.</th>
<th>d.</th>
<th>Mittel</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{C}_{27})</td>
<td>324</td>
<td>49,09</td>
<td>–</td>
<td>–</td>
<td>51,50</td>
<td>49,76</td>
<td>50,63</td>
</tr>
<tr>
<td>(\text{H}_{20})</td>
<td>29</td>
<td>4,39</td>
<td>–</td>
<td>–</td>
<td>4,65</td>
<td>4,09</td>
<td>4,37</td>
</tr>
<tr>
<td>(\text{Ba}^{#})</td>
<td>137</td>
<td>20,75</td>
<td>20,66</td>
<td>20,60</td>
<td>–</td>
<td>–</td>
<td>20,66</td>
</tr>
<tr>
<td>(\text{N}_{2})</td>
<td>42</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(\Theta_{8})</td>
<td>128</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>660</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>111</strong></td>
</tr>
</tbody>
</table>

### Tabelle 2

<table>
<thead>
<tr>
<th>Symbole</th>
<th>At.-Gew.</th>
<th>In 100 Th.</th>
<th>a.</th>
<th>b.</th>
<th>c.</th>
<th>d.</th>
<th>Mittel</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{C}_{18})</td>
<td>216</td>
<td>54,00</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>52,35</td>
<td>–</td>
</tr>
<tr>
<td>(\text{H}_{20})</td>
<td>20</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5,04</td>
<td>–</td>
</tr>
<tr>
<td>(\text{Ba}^{#})</td>
<td>40</td>
<td>10</td>
<td>9,63</td>
<td>9,88</td>
<td>9,92</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(\text{N}_{2})</td>
<td>28</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(\Theta_{8})</td>
<td>96</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>400</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Die Verbindung ist dennoch dem bereits beschriebenen halbsauren Baryumcholephäinat analog und hat die Formel \( \text{C}_{27}\text{H}_{29}\text{CaN}_{3}\text{O}_{8} \). Mit dieser Ansicht stimmen die Resultate der Analysen wie folgt.

<table>
<thead>
<tr>
<th>Theorie</th>
<th>Experimente.</th>
</tr>
</thead>
<tbody>
<tr>
<td>der Atome</td>
<td>p.C.</td>
</tr>
<tr>
<td>( \varepsilon )</td>
<td>324</td>
</tr>
<tr>
<td>( \text{H}_{20} )</td>
<td>29</td>
</tr>
<tr>
<td>( \varepsilon \text{a} )</td>
<td>40</td>
</tr>
<tr>
<td>( \text{N}_{3} )</td>
<td>42</td>
</tr>
<tr>
<td>( \Theta_{8} )</td>
<td>128</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Neutrales Calcium-Cholephäinat,</th>
<th>Halbsaures Calcium-Cholephäinat,</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{C}<em>{18}\text{H}</em>{20}\text{CaN}<em>{2}\text{O}</em>{6} ), At.-Gew. = 400.</td>
<td>( \text{C}<em>{27}\text{H}</em>{29}\text{CaN}<em>{3}\text{O}</em>{8} ), At.-Gew. = 563.</td>
</tr>
<tr>
<td>Theorie</td>
<td>Gef.</td>
</tr>
<tr>
<td>( \varepsilon )</td>
<td>54</td>
</tr>
<tr>
<td>( \text{H} )</td>
<td>5</td>
</tr>
<tr>
<td>( \varepsilon \text{a} )</td>
<td>10</td>
</tr>
</tbody>
</table>

The analytical data for these calcium salts were compared with Städelers's data for his calcium salt of bilirubin from human gallstones. At issue for Thudichum was the interpretation of Städelers's 9.1% CaO datum and his assumption that he was analyzing the neutral calcium salt. Thudichum reminds us of Städelers empirical formula (\( \text{C}_{18}\text{H}_{9}\text{NO}_{4} \)) published in 1861 in Frerichs's 2nd edition (120) of his famous Klinik der Leberkrankheiten (which did not involve analysis data for a calcium salt) and his subsequent formulas, \( \text{C}_{32}\text{H}_{18}\text{N}_{2}\text{O}_{6} \) for free bilirubin and \( \text{C}_{32}\text{H}_{17}\text{CaN}_{2}\text{O}_{6} \) 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 Städelers's formulas for biliverdin, Biliprasin, Bilifuscin (bilifuscin), and Bilihumin (bilihumin) would by necessity be incorrect (152):

In seinen Untersuchungen über den Farbstoff menschlicher Gallenstein stellte Städel ein Calciumverbindung des Bilirubins dar, welche ihm bei der Analyse 9,1 p.C. Calciumoxyxyl ergab. Von der Annahme ausgehend, dass diese Verbindung ein normales Neutalsalz 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 \( \text{C}_{32}\text{H}_{18}\text{N}_{2}\text{O}_{4} \) und substituirte \( \text{C}_{32}\text{H}_{15}\text{N}_{2}\text{O}_{6} \) als die Formel des freien Bilirubins, und \( \text{C}_{32}\text{H}_{17}\text{CaN}_{2}\text{O}_{6} \) 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 und 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 irrtümlich zu erklären.

Das von Städeler analysirte Bilirubat war offenbar das halbsaure Salz.

\[
\text{Theorie von } \text{C}_{27}\text{H}_{29}\text{CaN}_{2}\text{O}_{6} \quad \text{Städeler}
\]

<table>
<thead>
<tr>
<th>Atome</th>
<th>Theorie</th>
<th>Gefunden</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>324</td>
<td>–</td>
</tr>
<tr>
<td>H</td>
<td>29</td>
<td>–</td>
</tr>
<tr>
<td>Ca</td>
<td></td>
<td>9,1 p.C.</td>
</tr>
<tr>
<td>N</td>
<td>7,1</td>
<td>6,5 ..</td>
</tr>
</tbody>
</table>

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.

Thudichum also prepared zinc- and lead-Cholephäinat and carried out combustion analyses of each. The red-brown zinc salt, from ZnSO₄, was reddish-brown; the lead salt was from Pb(OAc)₂. Analyses for zinc, determined as ZnO, predicted a half-acid salt, C₂₇H₂₉ZnN₃O₈, composed of one molecule of neutral zinc Cholephäinat (C₁₈H₁₈ZnN₂O₄), one molecule of Cholephäin (C₉H₇NO₂), and two of H₂O (2×H₂O). In comparison a neutral salt of formula C₁₈H₁₆ZnN₂O₆ (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 zusammengesetzt ist, wie folgt.

\[
\text{1 neutrales Zinkcholephäinat ........... } \text{C}_{18}\text{H}_{16}\text{ZnN}_{2} \quad \Theta_{1}
\]

\[
\text{2 Wasser ....................... } \text{H}_4 \quad \Theta_{2}
\]

\[
\text{1 Cholephäin ......................... } \text{C}_{9}\text{H}_{7} \quad \text{N} \quad \Theta_{3}
\]

\[
\text{1 Mol. halbsaures Zn Choleph....... } \text{C}_{27}\text{H}_{29}\text{ZnN}_{3} \quad \Theta_{6}
\]

Mit dieser Auffassung harmoniren die Resultate der Analysen wie folgt.

\[
\begin{array}{cccc}
\text{Theorie} & \text{Gef.} \\
\text{der Atome} & \text{p.C.} & \text{a.} & \text{b.} \\
\text{C}_{27} & 324 & – & – & – \\
\text{H}_{29} & 29 & – & – & – \\
\text{Zn} & 65 & 11,05 & 12,03 & 11,30 \\
\text{N}_{3} & 42 & – & – & – \\
\text{Θ}_{6} & 128 & – & – & – \\
\end{array}
\]

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And from the lead salt analyses, with the lead being analyzed as lead oxide, as a basic Cholephäinat or as Cholephäin, the formula C₉H₇PbNO₂ was correlated, in which two hydrogens are replaced by one divalent lead. A formula corresponding to the di-silver salt of Cholephäin (C₉H₇Ag₂NO₂) seen above (152):
Diese Verbindung kann als ein basisches Cholephäinat oder als Cholephän aufgefasst werden, in welchem zwei Atome Wasserstoff durch ein zweidynamisches Atom Blei ersetzt sind.

<table>
<thead>
<tr>
<th>Theorie der Atome</th>
<th>p.C.</th>
<th>Gef. a.</th>
<th>b.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon_3$</td>
<td>108</td>
<td>29,34</td>
<td>–</td>
</tr>
<tr>
<td>H$_7$</td>
<td>7</td>
<td>1,9</td>
<td>–</td>
</tr>
<tr>
<td>Pb</td>
<td>207</td>
<td>56,25</td>
<td>58,38 57,91</td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>$\Theta_2$</td>
<td>32</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

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

Theorium 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$_2$O, 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$_2$SO$_4$ 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 I₂ led to a greenish-black resin, while reaction with Cl₂ in H₂O converted biliverdin to dirty yellow-colored flakes that were insoluble in H₂O and ether but readily soluble in alcohol. But when a saturated alcohol solution of biliverdin was subjected to a small blast of Cl₂ gas, the solution went colorless immediately and yielded chlorine-containing whitish-yellow flakes that were insoluble in H₂O 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 KCIO₃ during warming. Included were one soluble in CHCl₃, two in alcohol, but none in ether. (iv) When an alcoholic solution of biliverdin was heated with pure, moist Ag₂O, the pigment was converted to a purple-colored compound, Bilipurpurin (bilipurpurin). For the most part bilipurpurin remained insoluble and bound to Ag₂O, but it dissolved in ammonia to yield a green color and an excess of Ag₂O. 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 NH₄Cl with H₂O.

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 AgNO₃ 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 Ag₂O 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 H₂S, gave a clear yellow filtrate. After the alcohol had been removed, a yellow precipitate remained from which, after washing with H₂O 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 H₂O. 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 H₂O, insoluble in ether, but dissolved in aq. ammonia or KOH, and were precipitated by HNO₃ 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 PbO₂ 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 Ag₂O.
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$_2$O. The aqueous acidic liquor contained a fixed acid that formed a crystalline salt with Ag$_2$O.

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$_4$OH. 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):

\[
\begin{array}{cccccc}
 & a. & b. & c. & d. & e. \\
\hline
\varepsilon & – & 63,08 & 62,09 & – & 62,14 & 62,43 \\
\text{H} & – & 6,25 & 6,12 & – & 6,00 & 6,13 \\
\text{N} & 9,32 & – & – & 9,36 & – & 9,34 \\
\Theta & – & – & – & – & – & 22,10 \\
\multicolumn{6}{c}{100,00}
\end{array}
\]

Vergleicht man diese Befunde mit den das Bilirubin betreffenden Thatsachen,

\[
\begin{array}{ccc}
\text{Theorie in 100} & \text{Mittel der Analyse} & \text{Theorie} \\
\text{Bilirubin} & \text{Biliverdin} & \\
\varepsilon & 66,26 & 66,01 & 62,43 & 63,57 \\
\text{H} & 5,52 & 6,01 & 6,13 & 5,96 \\
\text{N} & 8,59 & 9,03 & 9,34 & 9,27 \\
\Theta & 19,63 & 18,95 & 22,10 & 21,20 & 116
\end{array}
\]

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 Städel er 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 \( \text{C}_9\text{H}_9\text{NO}_2 + \text{O} \rightarrow \text{C}_9\text{H}_9\text{NO}_3 \), 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. Städel er’s hypothesis, too, which might be formulated \( \text{C}_9\text{H}_9\text{NO}_2 + \text{O} + \text{H}_2\text{O} \rightarrow \text{C}_9\text{H}_9\text{NO}_3 \), 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 \( \text{H}_2\text{O} (2 \times \text{C}_9\text{H}_9\text{NO}_2 \rightarrow \text{C}_{18}\text{H}_{20}\text{N}_{2}\text{O}_5) \). 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):

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 \( \text{C}_8\text{H}_9\text{NO}_2 \), or one carbon atom fewer than in his bilirubin formula (152):

\[
\begin{align*}
\text{Atome} & \quad \text{At.-Gew.} & \quad \text{In 100 Th} & \quad \text{Mittel der Empirie} \\
\text{C}_9 & \quad 96 & \quad 63,57 & \quad 62,43 \\
\text{H}_9 & \quad 9 & \quad 5,96 & \quad 6,13 \\
\text{N} & \quad 14 & \quad 9,27 & \quad 9,34 \\
\text{O}_2 & \quad 32 & \quad 21,20 & \quad 22,10 \\
\text{151} & \quad 100,00 & \quad 100,00
\end{align*}
\]

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 \( \text{CO}_2 \): \( C_9\text{H}_9\text{NO}_2 + \text{O}_2 \rightarrow C_8\text{H}_9\text{NO}_2 + \text{CO}_2 \). 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):

\[
\begin{array}{ccccccc}
\text{Theorie} & \text{Analysen} \\
\hline \\
\text{der Atome} & \text{p.C.} & a. & b. & c. & d. & \text{Mittel} \\
C_72 & 864 & 60,20 & – & – & 61,33 & 63,06 & 62,19 \\
H_{77} & 77 & 5,36 & – & – & 5,68 & 5,8 & 5,74 \\
\text{Ca}_2 & 80 & 5,56 & 5,52 & 5,77 & – & – & 5,64 \\
\Theta_{18} & 288 & – & – & – & – & – \\
\hline \\
1435 & 119 \\
\end{array}
\]

*Thudichum* found that the %Ca fit best for a formula with a ratio nine biliverdins to two of Ca: \( C_{72}H_{77}\text{Ca}_2N_9\text{O}_{18} \), 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 Übereinstimmung 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.

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, \( C_{23}H_{27}\text{BaN}_3\text{O}_7 \), 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

\[
\begin{array}{cccc}
1 \text{ Mol.} & \text{neutrales Biliverdat} & . . . . . . & \text{C}_{16}H_{16}\text{BaN}_4\Theta_4 \\
1 \text{ Mol.} & \text{Biliverdin} & . . . . . . . . . . & \text{C}_8H_9\text{NO}_2 \Theta_2 \\
1 \text{ Mol.} & \text{Wasser} & . . . . . . . . . . & \text{H}_2 \Theta \\
1 \text{ Mol.} & \text{halbsaures Ba-Biliverdat} & . . . . . . . . . . & \text{C}_{23}H_{27}\text{BaN}_3\text{O}_7 \\
\end{array}
\]
### Theory of Atomes and Experiments

<table>
<thead>
<tr>
<th></th>
<th>Theorie</th>
<th>Experimente</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>der Atome</td>
<td>p.C.</td>
</tr>
<tr>
<td>( C_{24} )</td>
<td>288</td>
<td>47,52</td>
</tr>
<tr>
<td>( H_{27} )</td>
<td>27</td>
<td>4,45</td>
</tr>
<tr>
<td>( Ba )</td>
<td>137</td>
<td>22,60</td>
</tr>
<tr>
<td>( N_3 )</td>
<td>42</td>
<td>6,93</td>
</tr>
<tr>
<td>( O_6 )</td>
<td>112</td>
<td>18,50</td>
</tr>
<tr>
<td></td>
<td>606</td>
<td>100,00</td>
</tr>
</tbody>
</table>

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):

\[
\text{Lässt man das eine Atom Wasser aus der Berechnung weg, so stimmt die Theorie des Kohlenstoffs besser mit der Erfahrung, aber die des Baryums weniger gut.}
\]

### Summary

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 bilirubin (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\textsuperscript{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 Städelers new term “bilirubin” – behaved like an amide toward alkali because it released NH\textsubscript{3} and yielded a yellow or green pigment (150, 164):

\ldots 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 umkristallisirt; 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\textsubscript{3} and left briefly a red solution that turned green-yellow. Heating with Ba(OH)\textsubscript{2} or Ca(OH)\textsubscript{2} produced NH\textsubscript{3} and yielded Ba\textsuperscript{2+} or Ca\textsuperscript{2+} salts.

The Cholepyrrhin used had been isolated as a red-yellow pigment from human bile using the CHCl\textsubscript{3} extraction method of Valentiner (107, 108) and Brücke (109) and purified according to the latter by crystallization. Maly was apparently unaware that Städelers (135) and Thudichum (102) had to use more heroic methods to free

\textsuperscript{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 CHCl₃-acetic acid and thereby converted to a green color. After washing with H₂O (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 CHCl₃ 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 Stickstoffes 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 NH₃, der im ersten Falle entweicht, im zweiten als einfaches Ammonium salz sich vorfindet.

Das Biliverdin ist eine Säure, das 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 NH₃ 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 CHCl₃. 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 NH₃ being attributed to an impure sample of Cholepyrrhin; thus, in February 1868, Maly would write (166):


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 C₁₆H₁₈N₂O₃ for it (166):

\[
\text{Analyse.}
\]

I. 0,2770 Grm. aus Menschengallensteinen gaben bei der Verbrennung 0,681 Grm. CO₂ und 0,1545 Grm. H₂O.
II. 0,2734 Grm. Cholepyrrhin aus Ochesengallensteinen gaben 0,1532 Grm. Wasser.

Diese gibt in 100 Theilen Substanz:

<table>
<thead>
<tr>
<th></th>
<th>I.</th>
<th>II.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kohlenstoff</td>
<td>67,16</td>
<td>–</td>
</tr>
<tr>
<td>Wasserstoff</td>
<td>6,18</td>
<td>6,22</td>
</tr>
</tbody>
</table>

Diese Zahlen zeigen mit der Berechnung für C₁₆H₁₈N₂O₃ und mit den analytischen Mittelzahlen von Städelers:

<table>
<thead>
<tr>
<th>Bez. für</th>
<th>Mittel von Städelers (i.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOHlenstoff</td>
<td>67,13</td>
</tr>
<tr>
<td>WassErstoff</td>
<td>6,29</td>
</tr>
</tbody>
</table>

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. H₂SO₄ 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 H₂O, 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 NH₃ and potash (K₂CO₃) 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 HNO₃, 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% H₂O, 3% NaOH, 1% KOH), *Cholepyrrhin* gave, besides NH₃, a tarry compound with a decidedly aniline-like odor; however, the presence of the latter could not be confirmed.

*Maly* reinvestigated *Städelers Biliprasin* (135), which, as may be recalled, appeared along with biliverdin during the various manipulations that he used to purify bilirubin from gallstones. *Städelers* 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, Br₂ and I₂.

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 CHCl₃ 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 (H₂SO₃) – 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 H₂SO₃. Heating *Cholepyrrhin* in an aqueous or alcoholic solution of SO₂ 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.

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 PbO₂, and in two minutes the solution went over to green-brown; whereas, when the original solution was allowed to stand in air without PbO₂, 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 PbO₂ considerably shortened the time-consuming reaction taking place in air alone. In the presence of stronger oxidizing agents, biliverdin itself suffered further oxidation. Thus, KMnO₄ 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” (KMnO₄ also gives further oxidation products).

Maly was thus able to obtain the green pigment that he called Biliverdin from Cholepyrrhin by: (i) heating in CHCl₃-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 PbO₂ as well as Br₂ as an oxidizing accelerant (166, 167):
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, H₂SO₄, 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. H₂SO₄, the pigment dissolved to give a green color and was unchanged upon addition of H₂O, 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 CHCl₃ but soluble in CHCl₃ containing a few drops of alcohol, soluble in glacial acetic acid-CHCl₃, and also in glacial acetic acid with an especially beautiful color. It was not soluble in benzene or CS₂, poorly soluble in amyl alcohol and in CH₃CH₂I – but easily soluble in the latter two if a little ethyl alcohol is added (166, 167):

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 \( C_{16}H_{18}N_{2}O_{4} \). 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 \( C_{16}H_{20}N_{2}O_{5} \), which would give a value of 60% for C (166, 167):

**Analyse.**

II. 0,2905 Grm. Substanz einer anderen Darstellung gaben 0,1585 Grm. Wasser.
III. 0,3356 Grm. Substanz einer dritten Darstellung gaben mit Natronkalk gegluht 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:

<table>
<thead>
<tr>
<th></th>
<th>I.</th>
<th>II.</th>
<th>III.</th>
<th>IV.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kohlenstoff...........</td>
<td>63,74</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Wasserstoff............</td>
<td>5,97</td>
<td>6,05</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Stickstoff...............</td>
<td>–</td>
<td>–</td>
<td>8,77</td>
<td>8,74</td>
</tr>
</tbody>
</table>

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

\[
E_{16}H_{18}N_{2}O_{3} + \Theta = E_{16}H_{18}N_{2}O_{4},
\]

so wäre die Formel des Biliverdins \( E_{16}H_{18}N_{2}O_{4} \) und dieser entspricht die Berechnung:

<p>| | |</p>
<table>
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<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kohlenstoff...........</td>
<td>63,58</td>
</tr>
<tr>
<td>Wasserstoff............</td>
<td>5,96</td>
</tr>
<tr>
<td>Stickstoff...............</td>
<td>9,26</td>
</tr>
<tr>
<td>Sauerstoff...............</td>
<td>21,19</td>
</tr>
</tbody>
</table>

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

Nähme das Cholepyrrhin, wie Städelers angebte, auch noch ein Molekül Wasser auf:

\[
E_{16}H_{18}N_{2}O_{3} + \Theta + H_{2}O = E_{16}H_{20}N_{2}O_{5},
\]

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 (C₁₆H₂₀N₂O₅) 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 (C₁₆H₁₈N₂O₄) 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 (C₈H₉NO₂) for biliverdin, however, corresponded to one-half the Maly formula (171):

\[
\begin{align*}
\text{C}_{16} \text{H}_{18} \text{N}_2 \text{O}_4 &= \text{Bilirubin} + \Theta \\
\text{C}_{16} \text{H}_{20} \text{N}_2 \text{O}_5 &= \text{Bilirubin} + \Theta + \text{H}_2\text{O} + \Theta
\end{align*}
\]

In order to obtain a better combustion analysis of biliverdin, Maly prepared the latter from bilirubin in dilute aqueous Na₂CO₃ 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 NH₃ 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. CO₂ und 0·1452 Grm. H₂O.
2. 0·3693 Grm. eines anderen Präparates gaben 31·5 CC. feuchten N bei 15º C. und 27·35 Par. Zoll.
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.

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 ClCH₂CO₂H (62° C) in the presence of air for a few days turned the melt green; addition of H₂O 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 CO₂, 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. Na₂CO₃. 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):


<table>
<thead>
<tr>
<th>Gewichtsveränderung</th>
<th>0.0223</th>
<th>0.4681</th>
<th>0.4558</th>
<th>0.4458</th>
</tr>
</thead>
<tbody>
<tr>
<td>angewandtes Bilirubin</td>
<td>0.4558</td>
<td>Grm.</td>
<td>0.4458</td>
<td>&quot;</td>
</tr>
<tr>
<td>abfiltriertes Biliverdin</td>
<td>0.4558</td>
<td>&quot;</td>
<td>0.4458</td>
<td>&quot;</td>
</tr>
<tr>
<td>organische Substanz im Filtrat</td>
<td>0.0223</td>
<td>&quot;</td>
<td>0.0223</td>
<td>&quot;</td>
</tr>
<tr>
<td>gesammtes Biliverdin</td>
<td>0.4681</td>
<td>Grm.</td>
<td>0.4558</td>
<td>0.4458</td>
</tr>
</tbody>
</table>

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: C₁₆H₁₈N₂O₃ + O → C₁₆H₁₈N₂O₄, 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.

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$_2$O 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):

\[
\text{Sauerstoff} \quad \text{Kohlenstoff} \\
\text{Cholepyrrhin enthält } } 16,79 \text{ p.C.} \\
\text{Biliverdin } \text{“ } 21,19 \text{ “} \\
\text{Neuer Körper } \text{“ } 30,39 \text{ “}
\]

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.

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 CHCl₃ solution formed above was mixed with a CHCl₃ 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 Br₂ could be used to stop the oxidation at the blue stage (174). He believed he had found the means, using Br₂, to stop the progression and thus isolate pure pigments and said he would try to extend his research in that direction (166, 167):

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):

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 H₂ 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 CHCl₃ 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 (C₃₂H₄₀N₄O₇) was calculated from the assumption that two bilirubin molecules had together absorbed one molecule each of H₂ and H₂O. Maly named the new pigment Hydrobilirubin (hydrobilirubin) (176):


1. 0,2193 Grm. Substanz gaben 0,523 CO₂ und 0,1404 H₂O.
2. 0,2652 Grm. Substanz gaben 0,1646 H₂O.
3. 0,2262 Grm. Substanz gaben 0,1474 Platin.
4. 0,2483 Grm. Substanz gaben 0,5886 CO₂ und 0,1559 H₂O.
5. 0,2174 Grm. Substanz gaben 0,5142 CO₂ und 0,1347 H₂O.

Auf Procente bezogen:

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<tr>
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<th>1.</th>
<th>2.</th>
<th>3.</th>
<th>4.</th>
<th>5.</th>
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</thead>
<tbody>
<tr>
<td>C</td>
<td>64,89</td>
<td>–</td>
<td>–</td>
<td>64,65</td>
<td>64,50</td>
<td>64,68</td>
</tr>
<tr>
<td>H</td>
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<td>–</td>
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<td>6,87</td>
<td>6,93</td>
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<tr>
<td>N</td>
<td>–</td>
<td>–</td>
<td>9,22</td>
<td>–</td>
<td>–</td>
<td>9,22.</td>
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Diese Zahlen stimmen so gut unteneinander überein, dass die Reaction als eine sehr glatte bezeichnet werden muss. Die Substanz ist 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 H₂O auf 2 (Mol. ?) Bilirubin neben H₂, so würde der Körper C₃₂H₄₀N₄O₇ resultiren, nach der Gleichung:

\[ 2 \text{C}_{16}\text{H}_{18}\text{N}_{2}\text{O}_{3} + \text{H}_{2} + \text{H}_{2}\text{O} = \text{C}_{32}\text{H}_{40}\text{N}_{4}\text{O}_{7} \]
and this asks for:

Gefunden (Mittel)

<p>| | | |</p>
<table>
<thead>
<tr>
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<th></th>
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</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;32&lt;/sub&gt;</td>
<td>64.86</td>
<td>64.68</td>
</tr>
<tr>
<td>H&lt;sub&gt;40&lt;/sub&gt;</td>
<td>6.75</td>
<td>6.93</td>
</tr>
<tr>
<td>N&lt;sub&gt;4&lt;/sub&gt;</td>
<td>9.45</td>
<td>9.22</td>
</tr>
<tr>
<td>O&lt;sub&gt;7&lt;/sub&gt;</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

which agrees quite well with the obtained results. The new body, therefore, is formed by the uptake of hydrogen and water in double the amount of bilirubin (if not, as is perhaps more likely, the bilirubin formula is twice as large as usual) from bilirubin (if not, as is perhaps more likely, the bilirubin formula is twice as large as usual) formed and shall be called hydrobilirubin. 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<sub>2</sub> or ZnSO<sub>4</sub> in aq. NH<sub>3</sub>. 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):

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 not far removed from the composition of the hydrobilirubin, and would show that Scherer’s preparation was mostly pure. Other analyses consistently gave similar results. *)

*) 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.
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 H2O 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):


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 Nebenprodukte 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 Br2. 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 Br2 or I2. To accomplish such a transformation, described by Maly as surprisingly nice, Cholepyrrhin was allowed to stand in Br2 vapor mixed with moist air. This resulted in rapid darkening and yielded a compound no longer soluble in CHCl3 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 CHCl3 using a decently dilute solution of Br2 in alcohol. Dropwise addition of the latter into the former caused immediate darkening of the CHCl3 solution to a sap-green color. Careful addition led to a point where the CHCl3 solution was a clear, beautiful bright green, with the biliverdin formed remaining in the CHCl3-alcohol mixture. At this point Maly claimed that all
of the \textit{Cholepyrrhin} had been converted to biliverdin as the solution was stable for weeks (166, 167):

\begin{quote}
Ich habe erwähnt, dass es ausser Säuren und Basen noch eine dritte Reihe von Körpern gibt, 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 rein prachtvoll feuriges Grün zeigt \(^*)\). In diesem Momente ist alles Cholepyrrhin in Biliverdin übergegangen, und die Flüssigkeit kann wochenlang stehen, ohne sich zu verändern.
\end{quote}

\(^*)\) In diesem Gemenge von Chloroform mit nur wenig Alkohol bleibt das Biliverdin gelöst.

Thus, reaction of bilirubin with Br\(_2\) was viewed by \textit{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 \(\text{Br}_2\) took a few seconds.

Some 15 months after his July 9, 1874 presentation to the Austrian Academy, \textit{Maly}, then \textit{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 \textit{Annalen der Chemie und Pharmacie} (178). The subject was the treatment of bilirubin with halogens, especially \(\text{Br}_2\), and it drew a lambasting from \textit{Thudichum}, as will be noted later. Although \textit{Maly} had first believed that bilirubin was oxidized to biliverdin by reacting with \(\text{Br}_2\), 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 \(\text{Br}_2\) 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 \textit{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 \textit{Gmelin} HNO\(_3\) reaction). From a stable “blue step” produced by reaction with an appropriate amount of \(\text{Br}_2\), the solution was observed to run through the remainder of the color steps of the \textit{Gmelin} reaction, from red to yellow-brown. Based on his bromination experiments, \textit{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 begreif 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 \( \text{Br}_2 \) to bilirubin suspended in ether, and (ii) even better success with bilirubin suspended in alcohol-free \( \text{CHCl}_3 \) and addition of \( \text{Br}_2 \) in the same solvent. The elemental combustion analysis revealed that although the \( \% \text{C} \) varied considerably (35.51–47.83% among the six C,H analyses performed), the \( \% \text{H} \) (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 \( \text{C}_{32}\text{H}_{33}\text{Br}_3\text{N}_4\text{O}_6 \) (178):

\[
2 \text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_3 = \text{C}_{32}\text{H}_{36}\text{N}_4\text{O}_6 \\
\text{C}_{32}\text{H}_{36}\text{N}_4\text{O}_6 + 3 \text{Br}_2 = \text{C}_{32}\text{H}_{33}\text{Br}_3\text{N}_4\text{O}_6 + 3 \text{HBr}
\]

In order to reach the formula, Maly had to assume a doubling of his bilirubin “basic” formula (\( \text{C}_{16}\text{H}_{18}\text{N}_2\text{O}_3 \)) to \( \text{C}_{32}\text{H}_{36}\text{N}_4\text{O}_6 \). This, and the hydrobilirubin formula (\( \text{C}_{32}\text{H}_{40}\text{N}_4\text{O}_7 \)) derived from twice the original formula plus \( \text{H}_2 + \text{H}_2\text{O} \), induced him to rethink his formula for bilirubin. He concluded that the original “basic” formula could not be maintained and settled on \( \text{C}_{32}\text{H}_{36}\text{N}_4\text{O}_6 \) as the most appropriate (178):

\[
\begin{align*}
\text{C}_{32} & \quad 47.46 \\
\text{H}_{33} & \quad 4.08 \\
\text{Br}_3 & \quad 29.66 \\
\text{N}_4 & \quad 6.92 \\
\text{O}_6 & \quad 11.88
\end{align*}
\]

Maly’s formula is about as close to the correct formula (\( \text{C}_{33}\text{H}_{36}\text{N}_4\text{O}_6 \)) 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 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 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 “H₂” 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

![Diagram of Fraunhofer lines](image)

---

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.

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</thead>
<tbody>
<tr>
<td>17·5 II. $s$</td>
<td></td>
<td>32·0 I. $s$</td>
<td>33·1 IV. 2</td>
<td>29·8 III.</td>
<td>35·2 IV. 2</td>
<td></td>
</tr>
<tr>
<td>Faint continuous spectrum from 55 to 120</td>
<td>50·0 I. $s$</td>
<td>45·2 IV. $s$</td>
<td>41·7 I. 1·5</td>
<td>38·6 III.</td>
<td>41·5 III. 1·5</td>
<td></td>
</tr>
<tr>
<td>153·0 IV.</td>
<td></td>
<td>135·0 IV. $s$</td>
<td>52·8 IV.</td>
<td>56·0 III. 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The colours of the spectrum are approximately—red to 48, yellow to 52, green to 80, blue to 120, and violet beyond.
TABLE 19a.
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).

<table>
<thead>
<tr>
<th>Approximate Positions on Bunsen and Kirchhoff’s Scale.</th>
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<tbody>
<tr>
<td>Element</td>
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<tr>
<td>---------</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
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<tr>
<td>D₁</td>
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<td>D₂</td>
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<tr>
<td>E</td>
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<tr>
<td>b₁</td>
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<td>F</td>
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<tr>
<td>G</td>
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<tr>
<td>H₁</td>
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<tr>
<td>H₂</td>
</tr>
</tbody>
</table>

TABLE 19b.
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.

<table>
<thead>
<tr>
<th>Element</th>
<th>Wave-Length.</th>
<th>Scale Number.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kα</td>
<td>7685</td>
<td>1.10 metre</td>
</tr>
<tr>
<td>Liα</td>
<td>6705</td>
<td>,,</td>
</tr>
<tr>
<td>Hα</td>
<td>6562</td>
<td>,,</td>
</tr>
<tr>
<td>Liβ</td>
<td>6102</td>
<td>,,</td>
</tr>
<tr>
<td>Na</td>
<td>5892</td>
<td>,,</td>
</tr>
<tr>
<td>C</td>
<td>5662</td>
<td>,,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tl</td>
<td>5348</td>
<td>,,</td>
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<tr>
<td>C</td>
<td>5170</td>
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<td>Hβ</td>
<td>4861</td>
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<td>Sr</td>
<td>4607</td>
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<td>Ca</td>
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<td>Hγ</td>
<td>4101</td>
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<td>Kβ</td>
<td>4080</td>
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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 CHCl₃ 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. NH₃. 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 K₂CrO₇, 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 NH₃ 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 chromsauren 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.

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 verdünnterem erscheint
All this was from 1868, within a decade of von Steinheil’s building a spectro-
scope 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 hydrobili-
rubin, 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 phos-
phate to give a yellow, or a red-yellow or rose color, was placed in the spectro-
scope 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):

\[\text{Löst man etwas Hydrobiliirubin in verdünnten Alkohol, oder setzt man zu einer so}
\text{verdünnten alkalischen Lösung (in Ammoniak oder phosphorsaurem Natron u. s. w.) desel-
\text{ben, dass Säuren nichts mehr ausfällen, etwas Salz- oder Essigsäure bis zur sauren}
\text{Reaction, d. h. so weit, dass die Flüssigkeit die gelbe Farbe verliert und rothgelb oder}
\text{rosenfarbig wird, so zeigt sie in dünner Schicht (½ bis 2 CM.) vor den Spectralspalt gestellt}
\text{eine sehr lebhafte und markirte Absorption des Spectrums zwischen grün und blau, und}
\text{zwar bei meinem grössen Apparat (wenn Li bei 102,5; Na auf 120 und K-β auf 219,5 steht)
\text{innerhalb der Theilstriche 146 bis 160, oder allgemeiner ausgedrückt genau zwischen den}
\text{Fraunhofer’schen Linien b und F. Eben so bleibt es wenn die Lösung stärker sauer wird;}
\text{Ammoniak hingegen macht das Band verschwinden und lässt nur eine schwache diffuse}
\text{Absorption zwischen Grün und Blau, aber auf Zusatz von Säuren kehrt mit der röthlichen}
\text{Farbe das schwarze Band zurück. 148}

With the preparation of the zinc salt of hydrobiliirubin from aqueous NH₃, spec-
trum 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):

\[\text{Hingegen geben die ammoniakalischen Lösung des Farbstoffs, wenn sie etwas eines}
\text{Zinksalzes (auch Cadmium) gelöst enthalten, besonders schöne Bänder. Es genügt, der}
\text{stark ammoniakalisch gemachten Hydrobiliirubinlösung ein paar Tropfen von Zinkchlorür}
\text{oder –Sulfat hinzuzusetzen (wobei sich der entstandene Niederschlag leicht wieder löst)
\text{und diese Flüssigkeit vor den Apparat zu bringen. Oder man löst ausgefalltes}
\text{Hydrobiliirubinzink in Ammoniak und verdünnt. Beide Flüssigkeiten sind rosenroth und}
\text{geben ein durch Schärfe und Dunkelheit ausgezeichnetes Band, das gegenüber den sauren}
\text{Lösungen etwas nach links gerückt erscheint, daselbst bei 142 meiner Skale, also etwas vor}
\text{b, scharf abgegrenzt, nach rechts hin verschieden breit ist, je nach der Concentration der}
\text{Lösung, das aber immer am Dunkelsten von 142 bis 155 erscheint, d. i. von b an bis zur}
\text{Mitte des Spectralabschnittes b bis F. Die ganze Erscheinung ist mindestens eben so emp-
\text{findlich 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):

\[\text{Bringt man eine concentrirte Lösung vor den Spalt der Spectralapparate, so erscheint das}
\text{Spectrum vom violetten Ende her bis etwas zur Linie b völlig dunkel; beim Verdünnen hie148
\text{t sich der verdunkelte Theil allmählich auf und es bleibt schliesslich ein Absorptionsstreif}

2.10 The Emergence of Bile Pigment Spectroscopy: Colorimetry and Its Applications

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.

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 Br₂ 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, 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 NH₃ and a little ZnCl₂, 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 Li b 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 C₉H₁₁NO₃) 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.
C₉H₈NO₂), from human gallstones. From the latter “in caustic or carbonated alkali exposed to the air for some days” (186), he prepared biliverdin (C₈H₉NO₂). And to an aq. NH₃ solution of bilirubin, he prepared blue Cholecyanin by adding conc. HNO₃ dropwise; whereas treatment of the solid directly with fuming or conc. H₂SO₄ 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.

4. Urochrome, normal; by H₂SO₄ from lead precipitate. Dissolved in water and acid. Colour, yellow.
6. Omicholic acid; accompanies 5; soluble in NH₃. Dissolved in ether. Behaves like 5.
7. Uropittin, from extract or urine and urochrome by H₂SO₄. Dissolved in alcohol. Colour of solution, red.
10. Same as 9, more dilute. Colour, fine 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 10th 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 C₉H₇Br₂NO₂, was formed by exposing a weighed quantity of dry bilirubin in a watch glass to Br₂ vapor. When Br₂ 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×Br – 2×H₂ (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 C₉H₇Br₂NO₂ 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 Br₂, 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 C₁₈H₁₈N₂O₄, or close to Maly’s first proposed C₁₆H₁₈N₂O₃ (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 Br₂ (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 stoichiometrical 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 hydrobilibilirubin and Maly’s belief that he had transformed bilirubin into the coloring matter of urine, that there was a probable relationship to Jaffé’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 Jaffé [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 \( \text{C}_{32}\text{H}_{40}\text{N}_{4}\text{O}_{7} \) 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 spectrosopically 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 Städelers very early elemental combustion analyses of bilirubin found favor with Thudichum, who found it coincident with his own but at odds with Städelers 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 Städelers, and not a single element of any formula, can be derived from my analyses. . . . I hold the hypothesis of Städel 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) Malys final publication on bile pigments in 1875 and is illustrated in the following excerpts from 14 of the 40 very detailed points of Thudichums 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, Städel, in view of my researches, abandons all his former formula, 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 Br₂ 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 irrhümlich. 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 Hopkins55 and Garrod56 (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.**

<table>
<thead>
<tr>
<th></th>
<th>Urinary Products</th>
<th>Fecal Products</th>
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<tbody>
<tr>
<td></td>
<td>No. 1</td>
<td>No. 2</td>
</tr>
<tr>
<td>C</td>
<td>63·69</td>
<td>–</td>
</tr>
<tr>
<td>H</td>
<td>7·73</td>
<td>–</td>
</tr>
<tr>
<td>N</td>
<td>4·02</td>
<td>4·22</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th></th>
<th>Urobilin</th>
<th>Hydrobilirubin</th>
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<tbody>
<tr>
<td>Mean of above</td>
<td>Theory</td>
<td>Mean of Maly’s</td>
</tr>
<tr>
<td>results</td>
<td></td>
<td>results</td>
</tr>
<tr>
<td>C</td>
<td>63·58</td>
<td>64·86</td>
</tr>
<tr>
<td>H</td>
<td>7·84</td>
<td>6·75</td>
</tr>
<tr>
<td>N</td>
<td>4·11</td>
<td>9·45</td>
</tr>
<tr>
<td>O</td>
<td>24·47</td>
<td>18·94</td>
</tr>
</tbody>
</table>

[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 $\text{C}_{18}\text{H}_{25}\text{NO}_5$ (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 faeces urobilin and its chromogen.

When, as in certain cases of typhoid fever, the bile pigment is found in abundance in the faeces, 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 faeces, 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 faeces are urobilin-free, urobilin appears in the stools.

Thudichum had less patience with the 1894 publication of Jolles 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

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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 Städelers 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 Städelers formulas had gone unrecognized. After repeating Jolles’ I\textsubscript{2} reaction in CHCl\textsubscript{3} and with I\textsubscript{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\textsubscript{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\textsubscript{2} was not an oxidation but a substitution. He objected to Jolles’ not having isolated or analyzed the reaction products (187):

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\textsubscript{2}-promoted oxidation of bilirubin, finding a mismatch between Jolles’ product and authentic biliverdin (192):


He objected to Jolles’ use of unproven formulas and especially for an unproven reaction, bilirubin + I\textsubscript{2} “ biliverdin (187):

Unbegründete Formeln. Damit verschwindet die in der Abhandlung verschiedentlich wiederholte Formel, wonach eine Molekül sogenannten Bilirubins 4 Atome Jod und zwei Moleküll Wasser zur Oxydation zu Biliverdin erfordern und aufnehmen solle. Da die Reaction überhaupt nicht existirt, so müssen die den Pigmenten zugeschriebenen Formeln ungültig sein.
Thudichum was especially angered that Jolles would use a bilirubin formula (C_{32}H_{36}N_{4}O_{6}) ascribed to Maly and Städelier 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. Städelier had determined a different formula (C_{16}H_{18}N_{2}O_{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 Städelier hätten die Formel des Bilirubins als C_{32}H_{36}N_{4}O_{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 Städelier hatte dem Bilirubin die Formel C_{16}H_{18}N_{2}O_{3} beigelegt, dieselbe aber um 1870 verdoppelt, um dasselbe als eine sechsbasische 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\textsubscript{2} and Br\textsubscript{2} much earlier; (iii) Jolles’ belief that Choleletin is the end-product of the reaction of bilirubin with Br\textsubscript{2}, which Thudichum claimed to have shown to be invalid and that choleletin came from reaction with HNO\textsubscript{2} and not from Br\textsubscript{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\textsubscript{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 Städelier 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 Städelier’s earliest bilirubin formula (C_{16}H_{18}NO_{3}) as the only correct version. It matched his own. He disavowed the later Städelier formula (C_{16}H_{18}N_{2}O_{5}), which was based on the neutral calcium salt of bilirubin (C_{32}H_{36}N_{2}O_{6}, from 2(C_{16}H_{18}N_{2}O_{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_{20}N_{2}O_{6}Ca, based on Städelier’s C_{9}H_{9}NO_{2} for 3 × bilirubin + Ca(OH)\textsubscript{2}) theoretically has 7.1% Ca (Städelier found 6.5%); in contrast the neutral salt (C_{16}H_{20}N_{2}O_{6}Ca) yields 10% Ca – and thus cannot be identical to Städelier’s. Thudichum claimed that after his own investigations were published Städelier gave up on the second bilirubin formula in favor of its doubled formula (C_{32}H_{36}N_{2}O_{6}) without carrying out a single experiment or analysis. He noted that there is no correlation between the last Städelier formula and his
preparation and analysis and attributed Städelers changing formulas to desperation and the result of following an incorrect calcium analysis (187):


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):


To Jolles, the issue was not Städelers 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 I₂ could
not happen. In rebuttal, Jolles indicated that the results from treating bilirubin with Br₂ was a poor model for reaction with I₂, 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):


Thudichum viewed the green pigment as simply an iodinated substitution product of bilirubin, much as the reaction with Br₂ gave bromine substitution; Jolles disputed the first statement, not the last, and stood firm on the reaction with I₂ 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):

Jolles thus focused on Thudichum's dogma that I₂ cannot cause oxidation of bilirubin, only substitution (197):

Was nun Herr Thudichum in erster Linie bestreitet, ist die Thatscache, 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.

Jolles thus asked a fundamental question: why could Thudichum not see the possibility of several competing reactions taking place by reaction of bilirubin with I₂ (or Br₂ for that matter): oxidation, addition, and substitution? He queried correctly whether the reaction with I₂ might be more selective than reaction with Br₂, 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 einstheils Mischungen von Oxydations- und Substitutionsprodukt en waren, andererseits als bromirte Derivate von Oxydationsprodukten 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 Br₂ and I₂ is not generally permissible, especially when I₂ 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 substituierend wirkt, dies auch zweifellos beim Jod der Fall sein müsse. Ist ein solcher 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 substituierend wirkt, zumal in einer so außerordentlichen Verdünnung, worauf ja schon Kekulé zuerst ausführlich hingewiesen hat1)… 163

Jolles completed his work (197) with the publication of an experimental procedure for the oxidation of bilirubin to biliverdin by I₂, which he characterized by the equation C₁₆H₁₈N₂O₃ + 2 I + H₂O = C₁₆H₁₈N₂O₄ + 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 ZnCl₂, 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 C₁₆H₁₈N₂O₄ formula (197):

1. 0,1846 Grm. Substanz, bei 100° getrocknet, lieferten 0,3928 Grm. CO₂ und 0,0963 Grm. H₂O.
2. 0,1708 Grm. Substanz, bei 100° getrocknet, lieferten 14,4 Ccm. Stickstoff bei 728 Mm. und 20°.

<table>
<thead>
<tr>
<th>Bewertet für Biliverdin C₁₆H₁₈N₂O₄:</th>
<th>Gefunden:</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 63,58 %</td>
<td>62,76 %</td>
</tr>
<tr>
<td>H 5,96 „</td>
<td>6,27 „</td>
</tr>
</tbody>
</table>
| 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 I₂ (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 Oxydationsprodukt darstellt, und zwar ist dasselbe mit Rücksicht auf die Ergebnisse der Elementaranalyse, sowie der charakteristischen Eigenschaften des Körpers als Biliverdin anzusprechen.

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 I₂) 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.

Thudichum continued to object to Jolles’ published work, accusing him of first using the false oxidation of bilirubin by Br₂ as an analog of the purported oxidation by I₂ – 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 (C₁₆H₁₈N₂O₃) 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 (C₁₆H₁₈N₂O₃) 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 I₂) 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 Prozesse von Rödeler und Maly gemacht hatte, sind ebenfalls aus dem neuen Text weggelassen. Also z. B. anstatt C₁₂H₁₉N₄O₆, 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 C₁₆H₁₈N₂O₃. Er hat nun nicht etwa Bilirubin analysirt oder durch Verbindungen definiert, 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 I₂) were falsely called “determinations” and possessed no value whatsoever (191):


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 Br₂. 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):

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 Literatur 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 Omnicholin (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):


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.\textsuperscript{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 Literatur eingebürgert hat, halte ich seine Anwendung auf ein anderes Produkt nach den Gesetzen der litterarischen Ethik für unerlaubt.

\textit{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 \textit{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):


\textit{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 kristallisiertem Bilirubin verfasst, ohne dass dabei auch nur ein einziger Krystall zum Vorschein

\textsuperscript{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).
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 Küster’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 Zeugnis 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.

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: \textit{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; \textit{Berzelius} (1777–1848), who labored for nearly 40 years to isolate and purify bilirubin (\textit{Cholepyrrhin}, \textit{Gallenbraun}), biliverdin (\textit{Gallengrün}), and bilifulufin from bile – and for very different reasons dominated the field of chemistry; \textit{Tiedemann} (1781–1861) and \textit{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 HNO₃ that became the enduring and famous \textit{Gmelin} reaction (or diagnostic color test) for bilirubin in bile, urine, \textit{etc.}; \textit{Scherer} (1814–1869), who isolated a green pigment from bile and jaundiced urine and carried out one of the earliest elemental combustion analyses; \textit{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; \textit{Valentiner}, who while working in Friedrich Theodor von Frerichs’ lab in Göttingen in the 1840s introduced CHCl₃ extraction to isolate bilirubin and showed that it was probably identical to \textit{Virchow}’s hematoidin cited in 1847; \textit{Brücke} (1819–1892), who improved \textit{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; \textit{Städeler} (1821–1871), who isolated “purified” bilirubin and biliverdin and conducted C, H, N elemental combustion analyses that corresponded first to the formula \(C_{18}H_{18}NO_4\), then later to \(C_{32}H_{18}N_2O_6\) for bilirubin, and \(C_{32}H_{20}N_2O_{10}\) for biliverdin; \textit{Maly} (1839–1891), who also conducted C, H elemental combustion analyses of isolated bilirubin and suggested the formula \(C_{16}H_{18}N_2O_5\) for it, while the \%C, H, N of his biliverdin was fit to \(C_{16}H_{20}N_2O_5\); and \textit{Thudichum} (1829–1901), who carried out detailed isolations and combustion analyses to show that bilirubin had the (empirical) formula \(C_6H_3NO_2\), biliverdin had the formula \(C_6H_4NO_2\), 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 \textit{Städeler}, \textit{Maly}, and \textit{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 $\text{C}_{32}\text{H}_{36}\text{N}_{4}\text{O}_{8}$

(*Synonyms: Cholepyrrhin, Biliphaïn, Bilifulvin, Hæmatoidin*).

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 conjunctivae 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

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*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 $C_{16}H_{18}N_2O_5$. Ca. When this compound is dried in vacuo over sulphuric acid it is of a dark-green colour with a metallic 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 Städelier’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 Städelier and Maly from their analyses deduced for bilirubin the formula $C_{16}H_{18}N_2O_5$. 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 Städelier 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 Städelier and Maly’s formula on the one hand, and from that of Thudichum on the other.

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<tr>
<th>(Städelier and Maly.)</th>
<th>(Thudichum.)</th>
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<tr>
<td>$C_{16}H_{18}N_2O_5$ or $C_{32}H_{36}N_4O_6$</td>
<td>$C_9H_9NO_2$</td>
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<tr>
<td>Carbon</td>
<td>67.13</td>
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<td>Hydrogen</td>
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<td>Nitrogen</td>
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<td>Oxygen</td>
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$^1$Heintz, Poggendorff’s Annalen, Vol. LXXXIV, p. 106.


Quite apart from the remarkable concordance of the results of Städeler and of Maly, an examination of all facts bearing on the question 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.

When bilirubin is treated with pure dilute nitric acid (containing 20 per cent. of HNO₃) 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 (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 develop, 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 *Städel-Maly* formula for bilirubin was superior to his own and that *Städel*’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.*
Bilirubin: Jekyll and Hyde Pigment of Life
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Lightner, D.A.
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