# Bacteria and their dyes: Hans Christian Joachim Gram

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Coal-tar dyes, especially aniline dyes, are crucial to the history of bacteriology. They were discovered with the heightening of interest and enthusiasm for the chemistry of acids and bases and for that of Kekulé's benzene ring derivatives at the start of the twentieth century. In fact, until the dyes appeared, it had been truly difficult to carry out a successful *microbe hunt*. In this curious history along with the names of Weigert, Ehrlich, Koch, and many others here highlighted, we stress that of H.C.J. Gram. His tintorial differential method illustrates his present-day importance in taxonomy and research on the structure of bacteria. This method allows us to make organisms visible and to display their structure by revealing their chemical nature. However, today despite its long history, we do not yet know the *intrinsic* mechanism of Gram reaction.

**Key words:** *Bacterial Staining. H.C.J. Gram.*

Los colorantes derivados del alquitrán de hulla, especialmente los colorantes de anilina, han sido decisivos en la historia de la bacteriología. Su descubrimiento coincidió con el auge del interés y el entusiasmo por la química de los ácidos y las bases, así como los derivados del anillo bencénico de Kekulé, al comienzo del siglo XX. De hecho, hasta su aparición, fue realmente difícil realizar con éxito *la caza de los microbios*. En esta simpática historia destacamos, junto a los nombres de Weigert, Ehrlich, Koch y muchos otros, el de H.C.J. Gram. Su proceder tintorial ilustra su importancia actual en la taxonomía y la investigación de la estructura de las bacterias. Este método nos permite visualizar los microorganismos y mostrar su estructura al revelar su naturaleza química. A pesar de ello, y de su vieja historia, todavía hoy día no conocemos en profundidad el mecanismo *íntimo* de la reacción de Gram.

**Palabras clave:** *Tinción bacteriana. H.C.J. Gram.*

The most important thing in any invention is coincidence but most people do not happen to meet up with coincidences

Friedrich Nietzsche

## FROM ANILINE TO THE AZOIC DYES

The small size and transparency of microorganisms made them difficult to see even with the aid of a high-power microscope. In fact, this situation was due to some inadequacies of technique. It was truly impossible to identify bacteria until the nineteenth century, when thanks to the efforts of many scientists it was possible to create very sophisticated microscopes, and especially when dyes were used[1]. Staining facilitated the observation of a microorganisms by introducing differences in optical density or in light absorption between the microorganisms and its surround or between different parts of the same microorganism. Dyes used in staining microscopical specimens are *acid dyes* (v. gr.: orcein, safranin, methylene blue, and crystal violet), which color the nucleous and also attach to bacteria and cellulose (Table I) and *basic dyes* (v. gr.: various eosins, orange G, ponceau 2R, light green SF, and methyl blue) which color other cellular components. Each class contains dyes which attach directly (*direct* dyes) and those which attach to an intermediary (*mordant*) which is either applied before, or in the same solution as, the dye (v. gr.: carmine, hematoxylin, and celestin blue B[2]). The majority of dyes are *orthochromatic* in action, tha is to say, their action is direct and predictible under normal conditions; if it is a blue dye, it stains blue; if its a green dye, it stains green; and so forth. In the *metachromatic* staining, certain substances are stained in one color and others in another color by the same dye. The definition of metachromasia given by Paddy (1970) is "a characteristic rever-

TABLE I. **Some examples of *nuclear* stains**

| Stain | Use |
|---|---|
| *Direct nuclear stain* | |
| Lillie's ammonium oxalate crystal violet | Stain bacterial smears |
| LaCour's acetocarmine | Squashed cells for chromosomes |
| Gray's celestin blue B | Sections and small objects |
| *Indirect nuclear stain* | |
| Grenacher's alcoholic borax carmine | Wholemounts of small plants and animals |
| Ehrlich's acid alum hematoxylin | Plant and animal sectioned tissues |
| Johansen's safranin | Sections of plant and animal tissues |

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sible color change that any dye may undergo by virtue of a change in its environment not involving chemical reaction of the dye". Some metachromatic stains are methyl violet, brilliant cresyl blue, azure A, B, and C, touidine blue, new methylene blue, methylene blue, crystal violet, safranin, Bismarck brown, and basic fuchsin.

In the last quarter of the 19th century, research was performed on the chemical structure of the basic groups of dyes. Emil Fischer (1852-1919), 1902 Nobel Prize Winner in Chemistry, was especially outstanding in this respect. He paved the way for Verguin's discovery of fuchsin, Griess's find of aniline yellow, Manchester brown, methylene blue, Böttiger's Congo red, aniline violet, methyl violet, aniline black, Martius' yellow, auramines, benzopurpurines, rhodamines, anthrapurpurines, and the most significant of all dyes, allyzarine and Ehrenberg's indigo[3]. Johann Friedrich Adolf von Baeyer (1835-1917) is considered to be the *father* of colorants. A Nobel Prize Winner in Chemistry (1905) for his works on organic dyes and hydroaromatic chemicals, Baeyer[4] encouraged the development of organic and industrial chemistry. He discovered eosin, galein, cerulein, and nitrophenol, as well as indol and dioxindol. He revealed the reactions of the two indol dyes and of isatin with indigo, after having synthesized the latter. The Royal Society of London awarded him the Davy Medal in 1881. By 1885 he had developed a "strain" *(Spannung)* theory concerning the stability of certain cyclic organic compounds (valence tensions of cycle closures), as well as a centric formula for benzene. This entailed the introduction of the terms of configuration *cis* and *trans* to denote the geometric isomeres of unsaturated compounds (e.g., *cis*-maleic acid and *trans*-fumaric acid). Close collaboration between laboratories and industry allowed spectacular advances in synthetic colorants. The synthesis of these colorants was based on a well-established methodology. As a result, requests for patents began to abound after the pioneering groundwork had been laid in the industry of organic chemistry*.

The development of industrial organic chemistry was due greatly to the manufacture of alum gas and the growing need of metallurgical coal (Philippe Lebon and W. Murdoch). Coal, the *black gold,* was instrumental in promoting the industrial revolution. The residual products from the distillation of coal were used in research, especially the tars. Thus, Galle in German industry was parallel to the development of German power under the impulse of Otto von Bismark, minister to the King of Prussia, William I. Author of the unification of Germany in 1862, Otto von Bismark, with the victory at Sadowa in 1866, succeeded in situating Germany in the place of privilege previously occupied by Austria. Conqueror of France in 1871, was proclaimed in January in Versailles "Chancelor of the Empire". To isolate France, he constituted with Austria and Italy the Triple Aliance, converting Germany into a truely industrial and colonial power.

The increasingly high price of quinine as a result of the reduction of the source of quinine supply with the uncontrolled cutting of the *Peruvian cinchona* trees, stimulated search for effective substitutes for quinine in the management of fevers. One of the disciples of Liebig in Germany, August Wilhelm von Hofmann (1818-1892), researcher of aniline and its derivatives, broke ground in the colorant industry derived from hard-coal tar *(Teerfarben),* a thick black liquid obtained by heating the coal in the absense of air. In 1869 he published his results with carmine used as a bacterial staining agent[5]. Hofmann who was a professor at the London Royal College of Chemistry, was very interested in the applications of chemistry, especially in the field of medicine, wanting to manufacture natural drugs artificially and so suggested that quinine could be produced from the products of coal tar. This way, Europe could be free from her dependence on the remote tropics to provide quinine. His student from Manchester, William Henry Perkin (1838-1907), who had attended Michael Faraday's lectures (just as Faraday had attended Humphrey Davy's), tried to synthesize this drug oxidizing some derivatives of aniline with which he was then working[6]. Hofmann and Perkin sought to define the relationship between a substituted radical and the color change it produced in an artificial dye of known constitution[7]. Aniline was probably found by accidental contact with the skin in reducing fever[8]. In London he prepared the famous dye *mauvein*[9] (Tyrian purple) and developed the great aniline dye industry. During the Easter holiday of 1856, Perkin had mixed sulphuric acid with pure aniline in the presence of potassium dichromate and was about to throw out the resulting mixture as another failure when his eye perceived a purple reflextion in it. He added alcohol, which dissolved something in the preparation and it acquired a lovely purple colour "aniline purple"*. The French dyers acclaimed the new dye and called it *malva,* this period of history being known as the "Malva Decade". In the 60's Perkin achieved cinnamic acid by heating benzoic aldehyde with sodium acetate in the presence of acetic anhydride. The methylic and ethylic esters, with a pleasant odor are used in perfumery. Afterwards, Karl Graebe (1841-1927), Baeyer's disciple, was to synthesize allyzarin, colorant principle extracted from the madder root. In 1906, on the anniversary of the discovery of malvein, Perkins was to be knighted.

*Baeyer, Béchamp, Bretonière, Buchner, H. Caro, Coupier, Croissant, Doebner, Duisberg, Erdmann, Emil and Ottl Fischer, Fittig, J. and A. Gerber-Keller, Girard, Graebe, Green, Griess, Guyot, Heumann, Hofmann, Kékulé, Koechlin, Kostanecki, De Laire, De Lalande, Lauth, Levinstein, Martius, Meldock, Natanson, Nicholson, Nietzki, Perkin, Prud'homme, Rosenthiel, Roussin, Sandmeyer, Sapper, G. Schultz, Verguin, Vidal, Wichelhaus, Witt, Wurz.

* Perkin, along with B.F. Duppa, had discovered in 1858 how to synthesize glycine, and in 1860 how to synthesize tartaric acid.

Perkin's impressionable work extended throughout France thanks to Girard and De Laire, who working with aniline derivatives with diverse oxidizing agents achieved a new dye, magenta or rosaniline blue, whose derivative hydrosoluble sulfonate was prepared by Nicholson[10] in 1862. In 1863 Lighfoot obtained aniline black, the first *azoic* colorant (Bismark brown) and in the same year Hofmann presented iodine violet and in 1866 iodine green. Bardy later discovered dimethylaniline, from which he prepared in 1866 methylic violet, then called Paris violet, and in 1871 methylic green.

The consequences of the development of the colorant industry were many and very important. In the first place, there was a revolution in the industrial techniques and in the fabrication of basic and half finished products. Thus in 1890, R. Knietsch proposed the so called "contact method" for the preparation of sulphuric acid from $SO_2$ which competed with the classic "lead chamber", which was maintained thanks to improvements introduced*. This allowed the manufacture of indigo with a lowering of production costs. The subproducts obtained in the synthesis of the colorants reevaluated and prepared the development of the pharmaceutical industry thanks to the firms BASF, Kalle and MLB. In 1859, J. Kolbe prepared salicylic acid, whose antirheumatic properties were recognised in 1876 by Stricker. H. Dreser, the most brillant of the Bayer pharmacologists, obtained in 1899 by means of this ethylization procedures not only the discovery of acethylmorphin (heroin), but salicylic acethylacid (aspirin), proposing it as being effective as an antipyretic with analgesic action, contributing thus, in a decisive way, that the small colorant factory in Elberfeld was converted into a giant worldwide company (in 1915 it already had laboratories in Manchester, Vienna, Brussels, Vilna, Milan, Paris, Barcelona, Moscow, Melbourne, Bombay and Shanghai). L. Knorr synthesized in 1866 acetanilide, which with the name antipyrine (phenyl-dimetilpirazolone) was therapeutically widely used. E. Baumann succeeded in 1884 preparing from acetone and mercaptan, several hypnotics such as sulfonal, thiazol, and tetronal. Also, to this time belongs the isolation of cocaine, phenacetin, holocaine, salol or phenylsalicylate (discovered by Nencki), derivatives of theobromine, codine, xenophorm or bismuth salicylate and dermatol or basic gallate of bismuth.

Another consequence of the development of the colorant industry was the birth of synthetic perfumes. F. Tiedmann synthesized vanillin, an odorous compound, that up to that time was extracted from the husk of vanilla, which is used profusely in perfumery and especially in the art of culinary. En 1893, together with P. Krüger he prepared ionone, one of the most important compounds in perfumery. There were, what were without a doubt, notable consequences of a political, economical and juridical character. State intervention, above all during the 2nd half of the 19th century, is reflected in the diverse resolutions of a legislative character (e. gr.: the *1-1-1864* and the *Alkali Act,* in England) and the economical-administrative consequences, obliging for example the English textile industry to use natural alizarin to dye the uniforms, with an end to protect the countries own cultivation. The strong industrial competition on the international scene obliged the different countries to take a hard line customs stance in order to protect the interior market with unequal results. A determing factor was the state help with reference to the creation of research laboratories and university faculties to produce highly qualified personal. In Germany the laboratories of Liebig in Giessen, of Bunsen in Heidelberg, of Kékulé in Bonn and Hofmann in Berlin enjoyed renowned prestige.

The staining of bacteria followed the ordinary staining methods of histology. Probably Wilhelm Friedrich von Gleichen (1717-1783), *called* Russworm, was the first who employed carmine and indigo[11]. Carmine was also recommended by Goeppert and Cohn (1849), Corti (1851), Gerlach (1855)[12], Osborne (1856), and Clarke (1858), and hematoxylin by Waldeyer (1863) and Böhmer (1865)[13]. Ferdinand Julius Cohn (1828-1898) was especially pioneering in making histological stainings. He introduced the inclusion of pieces in soaps, celoindin and chloroform-paraffin and the examination of fresh and frozen preparations. In 1849 he prepared histological sections using the only vegetable colorant then available: carmine and hematoxylin[14]. These are members of a group of dyes called natural dyes. Unlike other natural dyes, carmine and also cochineal are derived from an animal source, a minute insect, *Coccus cacti,* which lives on spineless cacti. The dye is present as a purple sap in the females, which are harvested, dried, and pulverized to produce cochineal. This dye by itself has little affinity for tissue unless iron, aluminium, or some other metal is present. With the salt of one of these metals as a mordant staining will result. Alum cochineal, a commonly used form of this dye with mordant, is an efficient nuclear stain. The dye carmine is derived from cochineal by boiling the cochineal with a salt, usually alum, to produce a precipitate. This precipitate is insoluble in water, and before it can be used as a stain, it must be converted into a soluble compound such as ammoniacal carmine or acetocarmine. Hematoxylin can be regarded, in many respects, as the most important of the natural dyes. It was one of the first histological dyes and remains one of the most widely known and used dyes. Hematoxylin is extracted from the heartwood of longwood trees from South and Central America and the West Indies. The tree is *Caesalpina campechianum,* one of the legumes similar to acacia or cassia trees. Cristallized hematoxylin is not yet a dye, and its color must be allowed to develop by oxidation into hematein (color acid no relation to hematin, the colored constituent of red blodd cells). Used alone, hematein is only a weak and diffuse dye with little

*Glover, an acid manufacturer, made the method suggested by Gay-Lussac in 1860 for the recovery of nitric oxide possible, using the lead chamber process to make sulfuric acid.

affinity for tissues. A weak acid will not combine with nuclear elements in suficient quantity to produce efficient staining. Some form of mordanting (alum salts of aluminium, potassium, or iron) is required to form a base from this dye, which will then stain the acidic nuclear elements. The iron-mordanted form is one of the most valuable dyes for mitotic study. In the properly oxidized form it is an exceedingly pówerful dye with various shades of staining from purples, through blues, and into blues blacks. Others natural dyes are saffrom from stigmas of *Crocus;* indigo from plants of the genus *Indigofera;* berberine from barberry; orcein (a specific dye for elastin present in elastic fibers) and litmus from the lichens *Lecanora* and *Rocella;* and brazilin, chiefly from a few species of *Caesalpina,* a tropicopolitan genus of leguminous trees[15].

In 1875 Karl Weigert (1845-1904), called by his cousin Paul Ehrlich *"the master of the art of staining"*[16] (Fig 1), tested several simple colorants consisting of eosin (discovered by the chemist Heinrich Caro), fuchsin, methyl violet as well as methylene blue. Ehrlich subsequently discovered that dye[17]. Salomonsen (1877) stained bacteria by a watery solution of fuchsin to preserve the form of the microbes. His results support the concept of Ferdinand Cohn about the existence of different species of bacteria, against the school of Billroth who considered them as different types of the same species[18]. In his second bacteriological research, Koch (1877) greatly improved the methods of bacterial staining and laid the foundation of the technique employed to-day[19,20]. Robert Koch (1843-1910), 1905 Nobel Prize Winner in Medicine and Physiology, realizing the importance of getting the bacteria into a motionless state was the first to prepare thin films on cover-glasses and dried them. He dyed them with various stains, the most succeessful of which were methyl violet 5B, fuchsine, and aniline brown (Neubraun). Once dried in the air, he fixed the films in alcohol and covered them afterwards with a lamina so as to protect them and to make them lasting preparations. The stained preparations were finally mounted in potassium acetate solution or canada balsam[21]. He also succeeded in staining bacteria cillia. These had previously been figured by Ehrenberg (1838) in *B. triloculare* and by Cohn (1872) in *Spirillum volutans,* but by means of logwood extract and subsequent treatment with chromic acid, Koch was the first to obtain them in the stained state[22]. This technique was to be introduced by Ehrlich for blood films.

Ehrlich subjected the leucocytes to similar examination, using dried blood films, one cell thick and fixing them by heat, as in Koch's procedure for bacterial cultures. The films were stained with triacid and other dye mixtures which Ehrlich devised. He distinguished, in addition to basophils, specific lines of eosinophil (acidophil) and neutrophil granular leucocytes, as well as nongranular forms. This laid the foundation of morphological hematology and permitted an exact classification of the leucocytoses –and hence correct inferences as to underlying causes– and the differential diagnosis of

*Figure 1. Karl Weigert and Paul Ehrlich.*

leukaemias[23]. The use of methylene blue as a bacteria colorant was especially stimulated by Ehrlich (1854-1915), 1908 Nobel Prize Winner in Medicine and Physiology[24,25], who revealed his results in 1881. Ehrlich was familiar with methyl violet as a stain for deposit of amyloid in tissues, that was introduced by Cornil, and by Jurgens and Heschl, and with Zuppinger's use of aniline blue (triphenylrosaniline) to stain axis cylinders and dendritic processes of nerve cells. Ehrlich himself used Dahlia (monophenylrosaniline), a pruplish dye tried and rejected for his own purposes by Zuppinger, to stain selectively the cytoplasm of certain cells. widely distributed throughout the body. In 1877 he published the first paper on aniline dyes, *Contribution to the Knowledge of Aniline Dyes and their Application in Microscopical Technique* where he noted that while aniline dyes had found wide use in the textile industry they had been neglected by microscopists engaged in the study of animal tissues, even though the histologist was in a better position to make good use of them than the dyer of textiles[26]. It had been Weigert who introduced Ehrlich into the wondrous world of staining. Ehrlich took his Ph.D. in Medicine from Leipzig in 1878, at the tender age of twenty-four. His doctoral thesis concerned the analysis and value in Medicine of aniline staining. For a long time its was thought that Ehrlich's doctoral thesis had been lost until Professor Leonor Michaelis from Berlin found it in the Leipzig Medical School[27-29].

By using alkaline methylene blue and heating it, Koch succeeded in 1882 in staining *tubercle bacilli.* Nevertheless, bacteria thus treated seemed to show points or rods of dark color over a blue background, often indistinguishable from artifacts. At first, the solution to the problem seemed to be staining with methyl violet or with alcoholic fuchsin in aniline water, followed by an exposure to a solution of 3 % by volume of concentrated nitric or hydrochloric acid in water or in 95 % ethanol. The smear was then washed with water

and counterstained with methylene blue. But this did not work. Frank Ziehl (1859-1926) resorted to phenol with methyl violet instead of aniline water[30]. His technique was modified in 1883 by Friedrich Karl Adolf Neelsen (1854-1894). Neelsen introduced heated carbolfuchsin and a 15 % solution of sulphuric acid[31]. The merit of first staining tubercle bacilli by the methods used to-day was solely Ehrlich's, the modifications of Ziehl and Neelsen being trivial[32].

## FROM DYES TO DRUGS

Two years before Paul Ehrlich announced the discovery of Salvarsan[33,34], a chemist of the Technische Houchschule in Vienna, Paul Gelmo, had synthesized sulfanilamide (*p*-aminobenzene-sulfonamide). In 1919 Michael Heidelberger and Walter J. Jacobs, of the New York Rockefeller Institute made certain observations about the action of sulfanilamides. Although by 1908 the German dye industry was already committed to research into chemotherapeutics, the usefulness of sulfanilamide was to be confined to the augmentation of colorfastness in the red azo dyes for more than two decades. These dyes, which had been developed as early as 1900 by Heinrich Hörlein, Dressel, and Kothe, conferred on textiles a high resistance to washing and light, because of their intimate combination with wool-fibre proteins. This seemed a possible reason why they should adhere to the proteins of bacteria and so poison them. The investigation of these dyes (which presented a structural analogy with the components of acridine) formed part of the massive German research program set up to study the anti-bacterial activity of various dye substances which, for many years produced a long series of enigmatic results. Agents which exhibited antimicrobial activity *in vitro* (which had been demonstrated in some *azo* dyes in 1913 by Philipp Eisenberg) proved to be useless or highly toxic *in vivo*. Chemical manipulation undertaken in order to reduce the toxicity demonstrated in animal experiments invariably reduced or eliminated antimicrobial effectiveness. Although most German scientists followed this standard protocol of *in vitro* evaluation before the substance was tested *in vivo*, researches at the Research Laboratories of the I.G. Farben Industrie at Wupperthal-Elberfeld* initiated evaluation of each new agent by injecting it into laboratory mice infected with a virulent strain of hemolytic streptococcus. The most likely hint was given by Ehrlich's notion that dyes might be selective poisons. We know that trypan red and blue had had some success, and other workers had found that certain azo-dyes (those containing a group of two linked nitrogen atoms –N = N–) had some power for killing bacteria. In 1932, the young Director at Elberfeld, Gerhard Domagk, who was primarily a pathologist and bacteriologist and not a chemist[35,36], 1939 Nobel Prize in Mecicine, began testing a new azo dye which contained a sulfamyl group ($NH_2SO_2$). This substance, developed by I.G. Farben chemists, Fritz Mietzsch and Joseph Klarer, had recently been patented as Prontosil (*p*-2,4-diaminophenil-azo-benzene-sulfonamide). Gerhard Domagk discovered that Prontosil was unequivocably successful against the streptococcal infection in experimental animals, and this first trial ushered in the age of systemic antimicrobial therapy for infection[37]. The results of his research he put in his book *Ein Beitrag zur Chemotherapie der bakteriellen Infektionen*[38].

The discovery of the antibacterial effect of sulfamide determined the world-wide progress in the development of antimicrobial drugs during the last 5 decades. "Without Domagk there would not have been sulfamides, without sulfamides there would not have been penicillin and without penicillin there would not have been antibiotics", exclaimed Sir Alexander Fleming, 1945 Nobel Prize in Medicine. To Domagk is also due the introduction of TB 1, a drug belonging to the group of derivatives of thiosemicarbazone, synthesized by Behnisch, Mietzsch and Schmidt, and isoniazid (1951) synthesized by Offe and Siefken of the Scientific Laboratories of Leverkusen, in the treatment of TBC, and the use of the tumoral inhibitor E 39 (1956). The history of the discovery of sulfamides is the object of recollection in many books[39], appearing with particular precision in John Stewart Lawrence and John Francis's book, *The sulphonamides and antibiotics in man and animals* (London, 1953).

## HANS CHRISTIAN JOACHIM GRAM (1853-1938)

Hans Christian Joachim Gram[40,41] was born September 13, 1853 in Copenhagen and died there November 14, 1938 (Fig 2). Gram never used the names Hans and Joachim, but preferred to be called simply Christian Gram, abbreviating his name to Chr. Gram or C. Gram. He was the eldest of seven brothers and his parents were Frederik Terkel Julius Gram (1816-1871), a Common Law Professor, and Louise Christiane Roulund. He study in Regansen college, showing himself as a brilliant student, above all showing early his enthusiasm for the natural sciences. Throughout his life he cherised a love for, and knowledge of, plants. This knowledge would form the basis of his pharmacological interest, and his botanical training made him extremely familiar with the use of the microscope which put him definitely in the lead as compared with the majority of his contemporaries. The year his father died, he received his B.A. from Copenhagen Metropolitan School, where demonstrated a great love of sport; and from 1873 to 1874, served as a botanical assistant to the zoologist Johannes Japetus Smith Steenstrup (1813-1897)[42]. His interest in Medicine, fostered above all by Prof. Steenstrup, led him to earn his M.D. from

*In 1925, Badische Anilin und Soda-Fabrik, AG -BASF-having absorbed numerous companies formed the first trust of the colorant industry, with the name of *Interessen Gemeinschaft Farbenindustrie*, I.G. Farben; distmantled after the 2nd World War, the company is third ranking German chemical company today.

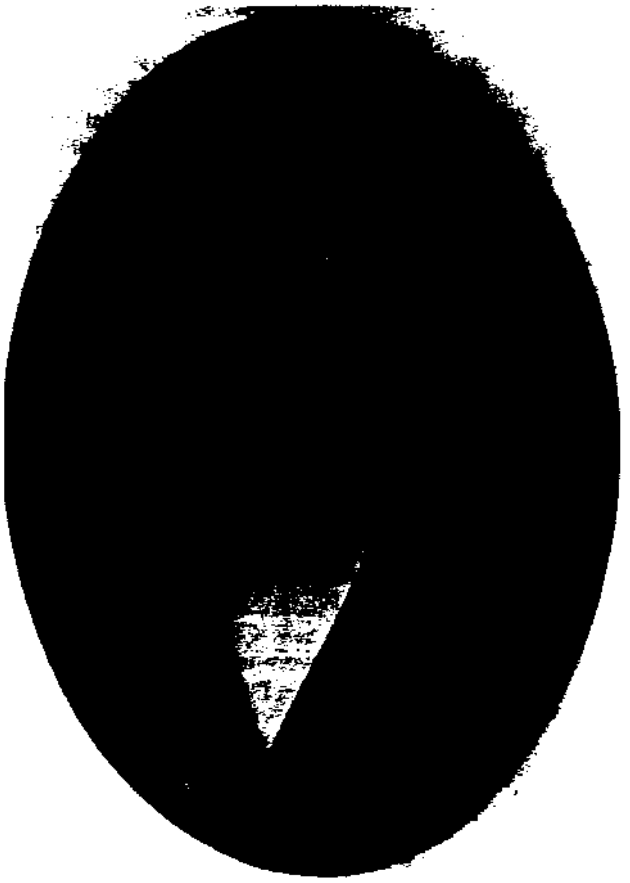

*Figure 2. H.C.J. Gram.*

the University of Copenhagen in 1878. In 1883 he gained the University Gold Medal by defending a Ph.D. thesis *"On the Size of the Red Blood Corpuscles in Man"* (previously studied by him in chlorotic patients) calling attention to their increase in size (macrocytes) in jaundice and in pernicious anemia (this age is especially rich in contributions to the knowledge of morphology and the quantitative counting of the elements of blood –Vierordt, 1869–, the dyes of Ehrlich, Perls, May, Giemsa, Grunwald and Pappenheim, and the first globular count in 1885 with Cramer's hematometer). He had the ingenious idea to examine the red blood cells in their own serum. He described the procedure of drawing blood into a capillary tube, sealing the ends of it, allowing the blood to clot, breaking the tube at the border of the clot and the serum, blowing out the serum on to a glass slide, and examining the erythrocytes.

The start of Gram in bacteriology comes from his participation in a theory-practical course on medical bacteriology in Cophenhague, by the design of Prof. Carl Julius Salomonsen in April 1883. Salomonsen (1847-1924) the year before had spent 15 days in Koch's laboratory in Berlin, learning the methods to colour bacterias especially the tuberculous bacillus[43]. Over the following two years he traveled throughout Europe and devoted himself to the study of pharmacology and bacteriology after taking his clinical training in several Danish hospitals.

## Gram's serendipity

Gram's was not a professional bacteriologist, but a physician and a teacher of general medicine. However, Gram's name is closely associated with a staining method which he had devised in 1884 and which allowed identification and counting of bacteria[44]. This discovery took place during his stay in Germany, where he did a postgraduate speciality with Karl Friedländer (1847-1887), Professor in the Stadtisches Allgemeines Krankenhaus and from 1881, editor of the influential journal *"Fortschritte der Medicin"* (assistant to Heidenhaim, he isolated the causing bacillus of lobar pneumonia in 1882 and he described obliterative arteritis in 1876) in the period when Koch and Ehrlich acquired fame for their use of aniline dyes for bacterial staining[45-48]. At this time also was the controversial debate between Friedländer and Albert Fräenkel about the etiological agent of cropous pneumonia[49]. In September 1881, Friedländer had began his investigation about pneumonia that would be preliminary published in 1882. However, his most important work appeared published in "Fortschritte der Medicin" on November 5th 1883, some two weeks after the arrival of Gram. As so often happens with great scientific findings, and according to an unconfirmed tradition, Gram made his discovery simply by chance[50]. In Friedländer's laboratory in Berlin, where he was working, Gram accidently spilled Lugol's iodine solution (Fig 3), an aqueous solution of iodinated potassium iodide[51], over sections (20 in total) of the lungs from fatal cases of lobar pneumonia[52], stained with methyl violet (it was believed at the time that the iodine acted as a mordant). When he washed them with absolute alcohol, he noted with surprise that the tissues became discolored. In relation to this, Gram wrote as follows:

"One case of cropous pneumonia with capsule-coccus. Here one finds very many which do not all lie in the cells of the exudate. They decolorize very easily in alcohol and, what is more, with and without treatment with iodine. From this case stems a great part of the cultures of Dr. Friedländer. Most of those from animals injected and exposed to infection behave in this fashion (mice, guinea pigs and a dog). Of these, I have investigated some 25 cases. Now and then, the cocci in the experimental animals remain colored after treatment with iodine but then they show no capsule formation; as everyone knows, capsules are always very difficult to demonstrate in cut preparations".

But in 19 case, the bacteria (Friedländer's pneumococci, which causes lobar pneumonia), on the other hand, kept their color. To tell you the truth, this only goes to show that an epoch-making discovery is sometimes made by taking advantage of a fortuitous happening. Gram's discovery was published as a small paper in Friedländer's journal under the title *"The Differen-*

*Figure 3. J.G.A. Lugol.*

*tial Staining of Schizomycetes in Sections and in Smear Preparations'*[53]. Friedländer immediately took up the method to a large extent in his investigations on pneumonia[54], and it is very interesting to see how Gram himself with great modesty says at the end of his paper: "With this method it is far easier to examine the schizomycetes, I have therefore published the method although I am aware that as yet it is very defective and imperfect; but it is to be hoped that also in the hands of other investigators will it turn out to be useful", to which the editor of the journal (Friedländer) quite contrary to what was the general custom, added: "Hierzu möchte ich mir die Bemerkung erlauben, dass ich die Gramsche Methode als eine ganz ausgezeichnete... kennen gelernt habe"[55]. The great themes related to Chr. Gram's contribution to the history of science, especially the discovery of his method of dying bacteria, as well as the vicissitudes and mischances about this discovery, are found developed in 9 letters to Salomonsen and were strictly analysed by Dr. Hans Lautrop[56].

Gram carried out studies abroad, in Switzerland, in Italy and in Strasbourg, where he learned new advances in pharmacology, experimenting in Oswald Schmiedeberg's laboratory, working on ptomaines sand digitoxin. On returning to Cophenhagen in 1885, where Gram abandoned forever bacteriology and dedicating full time to internal medicine and pharmacology. He became resident physician at the Kommunehospitalet, and was for some years district physician in Copenhagen. After some years of hospital practice, from 1891 to 1900, Gram taught Pharmacology at the University of Copenhagen, where he was appointed Lecturer in Pharmacology. There he made great contributions to the rational foundations of pharmacological science at a time when many therapeutical substances were being misused. He clearly explained all this as President of the Pharmacopea Commision (1901-1921). The birth of the new pharmacopea without a doubt is due a lot to Gram, especially when one refers to the way of presentation and administration of the new and numerous drugs that were emerging, coinciding with the great discoveries of that time that were being produced in mineral chemistry, organic and biologic, and especially in the progress of pharmacological methods. The extraordinary development of the pharmaceutical industry in Duisberg in 1888 and later in France, paved the way for finding new active principals (alkaloids and glucosoids), more effective and with better doses. Gram published his studies about a xanthin, teobromine (obtained from the seeds of cacao by Wodkressensky in 1832). In 1889, Gram married Louise Christiane Ida Lohse (1865-1900), who died eleven years later, victim of tuberculosis. He was appointed Full Professor of Pathology in 1900. He was devoted to teaching this discipline until his retirement in 1923, at seventy years of age. Afterwards, he rediscovered his earlier enthusiasm for the History of Medicine, and was engaged in the pursuit of his first love, botany, and interested himself in measures for preventing tuberculosis. Between 1902 and 1909, he published his four-volume work *Klinisk-therpeutiske Forelaesninger*. However, Gram's most outstanding feature as a researcher was his clinical work. Appointed Head of the Medical Department of the Royal Frederik's Hospital* in 1892, Gram acquired an interest in the clinical formation of young students. Furthermore, he maintained a private clinical practice, which attained renown. He was personal physician to the mother of Frederick VIII (Louise, died in 1903), of Frederick VIII and Alexandrine (wife of Christian X).

Gram's important work was recognized by the international scientific community. He was made Honorary Member of the Svenska Läkaresällskapet (1905), of the Verein für Innere Medizin (1907), and of the Dansk Selskab for Intern Medicin (1923). The University of Oslo (formerly Kristian University) named him *Doctor Honoraris Causa* in 1912. In the same year, the King awarded him the Dannebrog Commander's Cross, first class and in 1924 the Golden Medal of Merit. Gram's personality, considered totaly, as noted by some authors, sustained in the nobility of his character, methodical, personal charm, and extraordinary friendliness and cordiality; finally an expression of a singular intelligence and an altruistic spirit.

*It was built in 1757, in 1910 was replaced by the modern Righospitalet and now is the Museum for Applied Art.

## The puzzled question

Gram classified all known bacteria in accordance with whether or not they lost (Gram negatives) or retained their color (Gram positives). This subtle distinction is mainly due to the well-known basic differences in bacteria wall structure[57]. In general, there are correlations between the Gram reaction and certain important properties of bacteria. For instance, Gram-positive bacteria are more susceptible than Gram-negative bacteria to the bacteriostatic action of dyes, to halogens, to antibiotics, and to phagocytosis. They are more resistant to the bacteriolytic action of animal sera and to proteolytic enzymes like pepsin and trypsin. They are also more resistant to plasmolysis and have lower apparent isoelectric points than Gram-negative bacteria. Furthermore, Gram-positive bacteria are not versatile in their ability to synthesize aminoacids.

Gram never used contrast staining from Gram-negative bacteria as Weigert later would. Gram described his method as follows[58]:

"After having been dehydrated in alcohol, the preparations are immersed in the aniline-gentian violet solution of Ehrlich for 1 to 3 minutes (tubercle bacilli are immersed for 12-24 hours). The preparations are then placed in an aequeous solution of iodine-potassium iodide (iodine-1 gm., potassium iodide-2 gm., water 300 gm.) directly or after a rapid rinsing in alcohol. They are allowed to remain there for 1 to 3 minutes, during which time the color of the preparations are then completely decolorized with absolute alcohol. Further clearing is achieved with clove oil... Bacteria are stained intense blue while the background tissues are light yellow... I. The following forms of schizomycetes retain the aniline-gentian violet after treatment with iodine followed by alcohol: (a) cocci of croupous pneumonia (19 cases)... (b) cocci of pyemia (9 cases)... (k) tubercle bacilli (5 cases)... (1) anthrax bacilli (from 3 mice)... (m) various putrefactive bacilli and cocci... II. The following schizomycetes are decolorized by alcohol subsequent to treatment with iodine: (a) encapsulated cocci from cropous pneumonia (1 case)... (b) non-encapsulated cocci from cropous pneumonia (1 case)... (c) thyphoid bacilli (5 cases). The bacilli from typhoid fever are readily decolorized by alcohol either with or without prior treatment with iodine. Decolorization occurs even after the preparations have been immersed in stain for 24 hours."

In 1886, Karl Flügge (1847-1923), founder and co-editor with Koch of the influential journal *Zeitschrift für Hygiene*, wrote: "The method of Gram is mainly useful for the differential staining of bacteria in tissues and for the diagnostic differentiation of species." There have been many modifications of the original Gram staining technique, some of which were developed for specific purposes, and especially to produce a more rapid and sensitive method[59-67]. Peter Gray (1954) describes[68] most, if not all, of those invented up to the year 1951. J.W. Bartholomew (1962) as well as F.L. Tucker and J.W. Bartholomew (1962), and Emmel and Cowdry (1964) should be consulted by readers interested in the mechanism of Gram-staining and the variables which influence the results of staining[69]. Claudius proposed a modification of Gram's method by using and aequeous saturated solution of picric acid. On the other hand, Charles Jules Henry Nicolle (1886-1936), 1928 Nobel Prize in Medicine and Physiology, suggested a mixture of alcohol and acetone in a 5 to 1 proportion. Hucker's version consists in the use of crystal violet solution composed of 2 g crystal violet, 20 ml of 95 % ethanol, 0.8 g ammonium oxalate monohidrate, and 80 ml of distillated water and safranin 0 as counterstain[70]. The use of aniline dyes[71] called *basic colorants* (e.g., methylene blue, gentian violet, and basic fuchsin) therefore allowed a quicker staining of bacteria.

We know that the difference between Gram-positive and Gram-negative bacteria lies in the cellular wall and membrane. The highly complex morphology and specific architecture of these bacterial components were elucidated only by the use of many different methods such as freeze-fracturing or freeze-etching, ultra-thin sectioning, negative staining and shadow casting[72]. The Gram-positive bacteria can change their color with the addition of acetone or alcohol if the cellular wall is removed *after* staining stage but *before* the washing process. Although the chemical composition of the Gram-positive and Gram-negative bacteria are well known, as yet it is not clear why the cellular walls of the Gram-positive block the color extraction[73]. We seem, therefore that the intimate or exact mechanism of the Gram reaction for differentiating Gram-positive from Gram-negative bacteria remains still a partial mystery[74] in spite of the so many advances on the ultrastructural and biochemical properties of the bacteria cell wall[75-78] since 1884. The Gram-positive reaction is relatively rare. It occurs only among the bacteria, yeasts, and filamentous fungi. Very few biological structures are Gram-positives; they are those which are thought to be autoreproducing, and they include chromosomes of certain species, mitochondria, centrosomes, and centromeres.

Much work has been done on Gram's stain and a number of hypotheses have been advanced to explain its mechanism[79]. Unfortunately there is as *yet* no agreement either on facts or on hypotheses and the Gram stain is still not fully understood. In the past, most investigators attributed the reaction to differences in chemical composition (Guerbert et als, 1910; Schumacher, 1928; Williams et als, 1939), others to a difference of permeability between Gram-positive and Gram-negative bacteria (Fischer, 1897; Grudny, 1908; Benians, 1920; Bartholomew and Mittwer, 1952), especially to aminoacids, glutamic acid and lysine in particular. Gram-positive bacteria are distinguished by their ability to concentrate such aminoacids from the environment, while their synthesis occurs intracellularly in Gram-negative bacteria[80]. In particular, Gram-positiveness was attributed to the presence in Gram-positive bacteria of magnesium ribonucleate-protein complex[81,82]. Removal of this compound by treatment with the enzyme, ribonuclease, or a strong mineral acid (this is the basis of the widely used Feulgen reaction and of the HCl-Giemsa stain) renders the cell Gram-negative, and the cell so treated can reacquire its Gram-positive

property upon being soaked in a solution of the magnesium ribonucleate. This material can also be removed from Gram-positive cells by solution in bile to leave a Gram-negative cytoskeleton and redeposited to make the cells Gram-positive again. Gram-positivity was thought to be due to the presence of a phosphoric ester (Mitchell and Moyle, 1950) and later it was isolated as teichoic acid[83], by a difference in the tyrosine content of the pentose nucleoproteins (Hoffman, 1951), or by the carboxylic groups[84] of the dicarboxylic aminoacids (Gianni, 1952). At the present time emphasis is placed on the difference in cell wall structure and chemical composition, but it is not yet clear whether the cell wall *itself* is the substrate of the reaction or it acts *indirectely* by controlling permeability. Wiegel has suggested the elimination of the terms Gram-positive, negative, and variable (due to their equivocal nature), for the new terms Gram *type* positive, negative, and zero[85]. The first term would serve only to describe the results of the Gram staining reaction, whereas the second term would be used to indicate the classification of bacteria into taxonomically relevant groups. For instance, the recently introduced group *Archaebacterium*[86] lacks the typical peptidoglycan layers so it could be described by the term Gram *type* zero. On the other hand, the existence of microorganisms that have a rigid cell wall but do not contain peptidoglycan, has made some authors suggest a new division of the bacterial taxonomy[87]. Furthermore, it becomes clearer all the time, as pointed out by René Scherrer[88], that the Gram reaction is determined by the *physical* properties of each bacterial wall. The Gram reaction may be affected in technique and also by age of the culture, composition of the medium, autolysis, exposure to ultraviolet light and animal sera, and so forth. Despite the multiple advances in bacteriology and molecular biology, we continue without knowing the intimate mechanisms of the dying reaction discovered more than 100 years ago by Chr. Gram.

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medular cords with pernicious anemia; Friedrich Löffler (1852-1915) discovers the diphteria bacillus; G. Gaffky isolates the bacillus of typhoid; Mikulicz operates on a perforated thyphoid ulcer; Paul J. Möbius describes ophthalmoplegic migraine; Michael J. Rossbach describes hyperchlorhydria; Leopold Schroetter completes the description of venous thrombosis of the axillary vein; Ernst AGG von Strümpell describes polioencephalomyelitis; Ludwig Thudicum (1828-1901) publishes in London his studies on the biochemistry of the central nervous system; the use of antipyrine is introduced (Cf., Miroli AB. La Medicina en el tiempo. Librería "El Ateneo" Editorial. Buenos Aires, 1978:231-232.

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