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REVIEW ARTICLE

ALEXANDRE-LÉON ÉTARD

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Contribuciones a la química biológica

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RESUMEN

Alexandre-Léon Étard (1852-1919) fue un químico francés que estudió los alcaloides en general y la nicotina en particular. Junto con Cahours investigó la reacción de nicotina con azufre, sintetizó tetrapiridina y analizó su reacción con una variedad de reactivos. Los resultados indicaron que nicotina podía ser considerada una combinación de bipiridina e hidrógeno, cuya reacción con el azufre la transformaba en tetrapiridina y tetrapiridina. Esta última reaccionaba con el ácido nítrico generando isobipiridina. La reacción con selenio y otros reactivos condujo a la formación de colidina e isopiridina, y a proponer la posible fórmula del alcaloide. Étard también estudió el fenómeno de putrefacción e hidrólisis de los tejidos. Encontró que la liberación de nitrógeno señalaba el comienzo de la putrefacción, acompañada por el reemplazo de bacilos grandes por otros más pequeños. En la ausencia de aire, la putrefacción rompía la molécula de albumen y tenía lugar por una simple hidrólisis. Investigó las substancias que acompañaban a la clorofila presente en los vegetales y concluyó que la materia verde presente en las hojas contenía un núcleo fundamental, muy estable, que cumplía la función de absorción óptica conectada con el trabajo biológico. A este núcleo se podían conectar grupos químicos diferentes, generando así clorofilas que se diferenciaban por su composición, masa molecular, isomerismo, etc. y el papel que jugaban en las plantas vivientes. Étard y Vila separaron un derivado benzoílico presente en los productos de la hidrólisis de los tejidos, al que atribuyeron la causa del gran número de accidentes que ocurrían a consecuencia de consumir músculos.

Palabras clave: clorofila; hidrólisis; nicotina; putrefacción; tejido muscular.

ABSTRACT

Alexandre-Léon Étard (1852-1919) was a French chemist who studied alkaloids in general and nicotine in particular. Together with Cahours he investigated the reaction of nicotine with sulfur, synthesized tetrapyridine and studied its reaction with a variety of reagents. The results indicated that nicotine could be considered a combination of bipyridine and hydrogen, which upon reaction with sulfur transformed into tetrapyridine and thiotetrapyridine. The latter reacted with nitric acid yielding isobipyridine. The reaction with selenium and other reagents led to the formation of hydrocollidine and isopyridin, and the proposal of the possible formula of pyridine. Étard also studied the phenomenon of putrefaction and hydrolysis of tissues. He found that the release of nitrogen signaled the beginning of putrefaction, accompanied by the replacement of large bacilli by smaller ones. In the absence of air, putrefaction split the albumen molecule and proceeded by simple hydration. He studied in detail the substances that accompanied the chlorophyll present in vegetables and concluded that the green matter present in leaves contained a fundamental nucleus, very stable, that carried the function of optical absorption connected to the biological work. To this nucleus different chemical groups could be attached, giving place to chlorophylls that differed in their composition, molecular mass, isomerism etc. and the role they played in living plants. Étard and Vila separated a benzoyl derivative from the products of the hydrolysis of tissues and claimed that it was the cause of the many accidents that occurred during the eating of muscles.

Keywords: chlorophyll; hydrolysis; muscle tissue; nicotine; putrefaction.

INTRODUCCIÓN

Life and career (Olivier, 1910; Lebeau, 1911) Alexandre-Léon Étard (Figure 1) was born on January 5, 1852 in Alençon, Orne, the son of Alexander Louis Étard, an horticulturist and nursery gardener, and Anne Louise Royer. When he was three years old his father was hired by the Chilean government to build European style public parks and gardens in the country. At some time during his stay in Chile the child fell from a horse, broke a hip and was left with a coxalgia that would affect him all his life. In 1868 his father sent him back to France to complete his studies at the Lyceum in Alençon. In 1870 Étard and his family moved to Paris. While there, Alexander read an advertisement announcing a free course in chemistry to be given by Edmond Frémy (1814-1894) at the Museum of Natural History, a fortuitous event that would change his life and put him in the academic and scientific research track.

He so impressed Frémy that he took him to study and work in his laboratory learning experimental techniques. Eventually he received his degree of Licencie-ès-Sciences (1872) and Frémy sent him to become préparateur of the chemistry course given by Auguste Cahours (1813-1891) at the École Polytechnique. While in this position, he furthered his studies at the Sorbonne and in 1880 earned is doctorate in physical sciences, after successfully defending a thesis about the mechanism of oxidation and the introduction of an aldehyde group in hydrocarbons by means of the oxidation ability of chlorochromic acid (Étard, 1881a). His examiners were Paul Quentin Desains (1817-1885), Louis Joseph Troost (1825-1911), and Charles-Adolph Würtz (1817-1884).

In 1882 he became répetiteur adjunct at the École Polytechnique and in 1884 he was promoted to répétiteur titulaire. In 1885 he was appointed professor of general chemistry at the École de Physique et de Chimie Industrielle of the city of Paris and in 1899, *examinateur de sortie** at the École Polytechnique and Officier de l'Académie**. In 1901 he was chosen chief of services at the Institute Pasteur (Olivier, 1910; Lebeau, 1911).

In 1883 the Académie des Sciences awarded him the prestigious Jecker prize for his work in organic chemistry. In 1888 he was candidate in second line to a position in the chemistry section of the Académie des Sciences, in replacement of Henri Debray (1827-1888). In 1900, he was appointed chevalier of the Légion d'Honneur.

Étard died on May 1, 1919, as the consequence on an accident he suffered during a vacation in Tunis.

Scientific contribution

Étard wrote about 80 papers and books (Étard, 1894c, 1904, 1906, etc.), on the subjects of inorganic, organic, mineral, physical chemistry, and physiology. His most important biochemical contributions were on the subjects of nicotine, chlorophyll, putrefaction, and proteins. As customary of candidates to the Académie des Sciences, he wrote a booklet describing his research findings and their importance (Étard, 1894b). In addition to the subjects described below, he studied the chemistry of chrome, which led to the discovery of a reagent (Étard reaction) for introducing the group aldehyde in a hydrocarbon molecule (Étard, 1875, 1880, 1881a); the synthesis of hydrogen selenide (H_2Se) and hydrogen bromide (Moissan and Étard, 1880); the cupro-cupric sulfites, (Étard, 1881b, 1882ab); the solubility of salts and their mixtures (Étard, 1884, 1894d); the preparation of the carbides of yttrium and thorium and cast thorium (Moissan and Étard, 1896a, 1897b); etc.

In what follows, care must be taken to the fact that Étard used the old values of the atomic masses, i.e. O = 8, H = 1 and monoatomic, HO = water, etc.

** This academic palm was created in 1808 as an honor title reserved to members of the academy of the principal French universities.

*The examinateur de sortie is the officer that follows the students' progress during their schooling. He sits on the jury, which classifies the pupils in order of merit, at the end of the schooling at the École Polytechnique, and before their entrance in engineer and officers' training schools (École des Mines, École des Ponts et Chaussées, School of the

Nicotine and derivatives

In 1893 Cahours and Étard reported on the action of sulfur on nicotine (Cahours, and Étard, 1879). Heating gradually a mixture of 100 parts of nicotine and 20 of sulfur to 140 ^{0}C and more resulted in the release of large amounts of hydrogen sulfide, showing that sulfur had dehydrogenated nicotine. When the temperature reached 160^0-170 ^{0}C the mass became fluid and assumed a chrome green color. Upon cooling and left to itself for several days, the mixture deposited a large amount of prismatic sulfur yellow crystals, several millimeters long. These crystals were separated by expression, purified by dissolution in an acid, reprecipitated and crystallized from boiling alcohol, and decolorized with carbon black. The purified yellow crystals were sparingly soluble in cold alcohol, benzene, and ether, insoluble in water, and showed no action on litmus paper. They melted at 155 ^{0}C and on cooling became a yellow resinous mass. Upon distillation they decomposed releasing hydrogen sulfide. Elemental analysis indicated that their composition corresponded to the formula $C_{40}H_{18}N_4S_2$. Cahours and Étard named the new compound *tetrapyridine* and reported its reaction with a variety of reagents such as HCl, sulfuric acid, alkalis, platinum dichloride, mercuric chloride, picric acid, gold chloride, and potassium dichromate. All these results suggested that nicotine could be considered a combination of bipyridine and hydrogen, and that the reaction of sulfur with two molecules of nicotine transformed the latter into tetrapyridine and thiotetrapyridine, as per the following reactions:

$2C_{20}H_{14}N_2 + 4S_2 = 4H_2S_2 + C_{40}H_{20}N_4$
nicotine tetrapyridine

$C_{40}H_{20}N_4 + 2S_2 = H_2S_2 + C_{40}H_{18}N_4S_2$
tetrapyridine thiotetrapyridine

Cahours and Étard wrote that the dehydrogenation by sulfur, as illustrated by these equations, was not the limit of the reaction. Employing a excess of this reagent and heating to higher temperatures resulted in the release of large amounts of H_2S and formation of a new unidentified compound (Cahours and Étard, 1879).

In a following publication Cahours and Étard reported that the reaction of thiotetrapyridine with diluted nitric acid produced nicotinic acid nitrate, a compound crystallizing as white needle crystals melting at 228^0-229 ^{0}C (Cahours and Étard, 1880a). They also found that metals such as copper, mercury, and silver, were able to desulfurize thiotetrapyridine, and yield colorless basic oil, boiling between 274^0 and 275 ^{0}C and becoming colored in contact with air. This oil had a relative density 1.1245 at 15 ^{0}C, did not solidify even when cooled to -20 ^{0}C, and was easily soluble in alcohol, ether, and HCl. The resulting chlorhydrate allowed separating the free base with either KOH or ammonia. Elemental analysis of the base indicated that its composition corresponded to the formula $C_{20}H_{10}N_2$, identical with that of bipyridine but having different properties. For these reasons Cahours and Étard named it isobipyridine. Cahours and Étard described the preparation and properties of a variety of derivatives of isobipyridine, among them chlorhydrate, chloroplatinate, chloromercurate, and ferricyanhydrate (Cahours and Étard, 1880a).

In 1864 Carl Huber reported the preparation of a bromine derivative of nicotine to which he attributed the formula $C_{20}H_{13}NBr_5$, that is, dibrominated nicotine bromohydrate. He prepared it by reacting bromine with an ethereal solution of nicotine (Huber, 1864). Cahours and Étard thought that since bromine reacted with ether, Huber's derivative instead of being the product of the replacement of hydrogen of nicotine by bromine was actually the product of the reaction of the *ether* with nicotine. For this reason they decided to repeat the reaction replacing the ethereal solution of nicotine by an aqueous one and so fix the bromine by

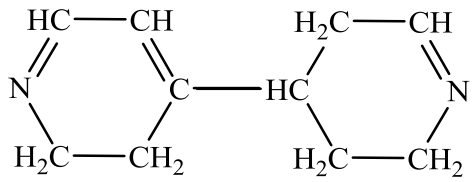
addition on the pyridine rings known to exist in the nicotine molecule (Cahours and Étard, 1880bc). They took a solution of 1 part of nicotine in 50 of water and reacted it in the ratio 4 atoms of bromine per 1 mole of nicotine. They observed the formation of an abundant yellow flocculent precipitate. The temperature was then raised to 65^0 to 70 ^{0}C by means of a stream of steam and afterwards the solid separated by filtration. Upon cooling, the filtrate precipitated abundant crystals. Interesting enough, the solid portion also yielded the same crystals after being heated with steam at 70 ^{0}C. All the crystals appeared as red and fine needles, stable at room temperature, acting on polarized light, and having formula $C_{20}H_{14}N_2Br_4$. Heated in a glass tube they released bromine and hydrogen bromide. These results implied that Huber's compound was the bromohydrate of this derivative (Cahours and Étard, 1880bc).

A final publication reported the synthesis of a new compound resulting from the action of selenium upon nicotine (Cahours and Étard, 1881). According to Cahours and Étard, mixing nicotine with selenium at 240 ^{0}C did not show a reaction, only dissolution of the element in the liquid, followed by its precipitation upon cooling in the form of microscopic vitreous spheres, colored black. If the contact persisted for a long time while maintaining the mixture boiling, a lively reaction took place accompanied by precipitation of white flaky crystals, containing selenium and ammonia. Cahours and Étard wrote that they did not analyze completely these crystals because they had a fetid smell and provoked long lasting headaches. Part of this material was distilled, passing a liquid fraction boiling between at 150^0 to 300 ^{0}C and leaving a tarry paste. Purification of the liquid phase indicated that it was a *hydrocollidine* of formula $C_{16}H_{13}N$. Hydrocollidine was described as amber liquid, limpid, lighter than water, boiling at 205 ^{0}C, having a penetrating aromatic smell, and a burning taste. It was completely soluble in alcohol, ether, diluted acid, and insoluble in water. Cahours and Étard described the preparation and properties of the chloraurate and chloroplatinate derivatives. They believed that selenium acted on nicotine forming two principal substances: hydrocollidine and *isopyridin*, accompanied by unidentified resinous materials (Cahours and Étard, 1881).

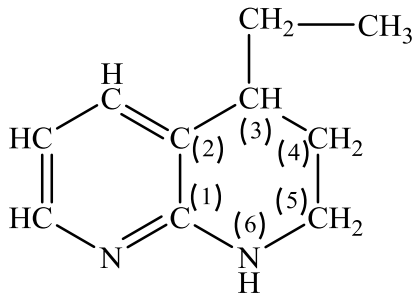
Étard wrote that although the oxidation of nicotine normally yielded nicotinic acid, he had found that mercuric oxide did not produce this result. Étard heated the nicotine to 240 ^{0}C and added the mercuric oxide in small quantities. The energetic reaction gave off water and metallic mercury. Purification of the resulting material with hydrogen sulfide and alkali produced a brown black matter of composition, by weight, 71.5% carbon, 5.4% hydrogen, and 16.7% nitrogen, corresponding to the formula $(C_{10}H_9N_2)_2O_2$, which Étard named *oxytrinicotine*, a compound comparable to the thiotetrapyridine described above, $(C_{10}H_9N_2)_2S$.

In a following experiment, Étard heated a mixture of 5 g of nicotine with 5 g of red phosphorus and 60 g of fuming HI in a sealed tube at 260^0-270 ^{0}C for 10 hours and noticed the liberation of hydrogen and the formation of a crystalline product, which he believed was a periodide. The crystals were treated with KOH and the liberated oily bases fractionated. This process yielded a small amount of hydrocollidine boiling at 205 ^{0}C, nicotine boiling at 244 ^{0}C, and *hydronicotine*, an almost odorless oily liquid, levorotatory (α_D = -15^{0}40'), boiling at 263^0- 264 ^{0}C, specific gravity 0.993 at 17 ^{0}C, and soluble in water. This hydronicotine was the first hydrogen derivative known of the alkaloid (Étard, 1883).

The isopyridin discovered by Cahours and Étard led many chemists to propose that nicotine was a hydride of one of the known dipyridyl isomers, for example:



Étard did not believe that all the nitrogen atoms of nicotine were tertiary and that nicotine was not a mono substituted product of pyridine (Étard, 1893b). To him, there was another formula that gave a correct interpretation of the all the experimental evidence:



He justified his formula with the following arguments: (a) nicotine was optically active by carbon (3); this activity was maintained in the hydride and disappeared in isodipyridin; (b) nicotine yielded an hexahydride; (c) oxidation attacked the nicotine in carbons (1) and (2) producing only one mono substituted acid (nicotinic acid); and (d) treatment of nicotine with HCl or HI never yielded -CH_2Cl or -CH_2I, meaning that it did not contain the group =N-CH_2. It remained to prove that nicotine contained one =NH group, fact that was proved in a following publication (Étard, 1893bc). Étard reported the preparation of diacetyl nicotine, as primary proof of this assumption. He heated to 150 ^{0}C, in a closed vessel, an equimolar mixture of anhydrous nicotine and acetic anhydride and noticed that the liquid became colored brown without an increase in the internal pressure. He then treated this liquid with a concentrated ethereal solution of NaOH and noticed the formation of three liquid layers, one of ether containing a very small amount of pyridine bases, a second of aqueous NaOH, and an intermediate phase, insoluble in ether, that was not nicotine. The latter phase was treated with potassium chloride. Analysis of the resulting material indicated that it was a chloroplatinate of acetyl nicotine, containing, by weight, 26.5% carbon, 3.6% hydrogen, 30.3-30.5% platinum, and 27.4-27.5% chlorine, corresponding to the formula $C_{14}H_{21}N_2O_3HCl.PtCl_4$ (Étard, 1893b).

Étard believed that his results indicated that nicotine combined with acetic anhydride by simple addition, generating a quaternary hydride, the acetylated derivative, of composition [($C_{10}H_{13}N = N(C_2H_5O)_2.OH$] (Étard, 1893b).

Étard wrote that in his previous publication he had remarked that most chemists considered nicotine as a base having *two* tertiary nitrogen atoms -N= (Étard, 1893c). Their best proof of this assumption was that nicotine did not react with acid chlorides, such as benzoyl chloride, to yield benzoylnicotine accompanied by release of HCl. A result of this nature would contradict Étard's previous claim that nicotine actually contained a *secondary* nitrogen atom -NH- and a *tertiary* one (Étard, 1883). Hans Will had already reported that benzoyl chloride gave with nicotine a compound of simple addition having the formula $C_{10}H_{14}N_2.2C_7H_5OCl$ (Will, 1861). Étard did not check this finding but wrote that he had already observed that nicotine formed with benzoyl chloride a benzoic derivative. He now reacted 16 g f dry nicotine with an excess of freshly distilled benzoyl chloride and noticed that no reaction

occurred at room temperature. Heating to the boiling point of the chloride produced a live reaction with abundant release of HCl, according to the following equation

$C_{10}H_{14}N_2 + C_7H_5OCl = HCl + C_{10}H_{13}N_2.C_7H_5O$

Étard described benzoylnicotine as a colorless, fetid, slightly viscous material, insoluble in water, and soluble in an excess of diluted HCl. Its chloroplatinate was a yellow crystalline material containing, by weight, 42.5-42.7% carbon, 4.0-4.1% hydrogen, and 20.7-21.0% platinum, corresponding to the formula $[C_{10}H_{13}N(C_7H_5O).HCl]_2.PtCl_4$. According to Étard these results indicated clearly that acid chlorides reacted with nicotine and that the molecule of the latter contained a hydrogen atom joined to nitrogen one (Étard, 1893c).

Putrefaction

Étard wrote that organized tissues left alone experimented a deep transformation caused by microorganisms and similar in every aspect to the process known as putrefaction. These phenomena were important from the viewpoint of conservation of labile substances and also for understanding the composition of the albuminoidal substances, which were the essence of living beings (Étard, 1894b). In 1872, the French physician and biochemist Armand Gautier (1837-1920) and the Italian chemist and toxicologist Francesco Selmi (1817-1881) reported independently that the rotting of corpses was accompanied by the formation of poisonous alkaloids (*ptomaines*) strongly resembling vegetable alkaloids and which in the case of legal inquiries could cause serious errors. Selmi thoroughly studied these cadaveric alkaloids and established how they could be distinguished from ordinal alkaloids. In 1882 Gautier published an historical review of this discovery and mentioned that around 1872 he had reported that well-washed fibrin blood, abandoned to putrefy in water, liquefied and generated albumin, casein, and butyric acid, accompanied by a small amount of fixed and volatile alkaloids (Gautier, 1874, 1882; Selmi 1876). In the same year (1882) Gautier and Étard published the first results of their study of the mechanism of the putrefaction of proteinic matter (Gautier and Étard, 1882a). In this project, they stored large amounts of beef, horse, and fish meat in watertight closed oak barrels and glass carboys and abandoned them to their own during the hot days of the summer of 1881. In this manner they could easily observe what was taking place in the inside of the containers, take samples for microscopic analyses, collect the gases formed, etc. They found that in the beginning the muscles of beef and horse became acid and odorless and then turned acid and began to ooze a clear syrupy liquid that seemed to indicate the beginning of the digestion of the muscular flesh by the appropriate ferment. The liquid was found to contain 21 to 22 g per liter of albumin coagulable by heat, and a very small portion of casein. Lactic and butyric fermentation followed, accompanied by release of gas containing increasing amounts of CO_2 and traces of hydrogen sulfide and phosphorus derivatives. No hydrocarbons were ever produced. Large bacilli were clearly present. The release of CO_2 seemed to indicate the transformation of a carbohydrate into lactic and butyric acids according to
$2C_6H_{12}O_6 = 2C_3H_6O_2 + C_4H_8O_2 + 2CO_2 + 4H$ (hydrogen was still believed to be monatomic).
This was the phenomenon that preceded true putrid representation (Gautier and Étard, 1882a). The appearance of nitrogen signaled the beginning of putrefaction and was accompanied by the replacement of the large bacilli by smaller ones, which attacked the albumin molecule on the uric side, causing the release of ammonia and CO_2, which turned the medium alkaline. Sometime later, the gas release stopped but the transformation of the flesh continued. At the end of a few months the liquid was free of albumin and casein and the mass of the molecule had become a mixture of extractable materials soluble in alcohol, containing some trimethylamine, fatty matter, oxalic acid, phenol, guanidine, indole, skatole,

xanthine, and organic alkalis. It was clear that in the absence of air, putrefaction split the albumin molecule and proceeded by *simple hydration* (Gautier and Étard, 1882a).

In a following paper Gautier and Étard added that putrefaction was accompanied by the formation of acetic, butyric and succinic acids, and by large amounts of hemiproteins and glycoproteins, leucines, tyrosine, and other aromatic compounds (Gautier and Étard, 1882b). The putrefaction of fish meat was accompanied by the formation of large amounts of a crystalline substance of formula $C_{11}H_{22}N_2O_6$. Gautier and Étard mentioned that the liquid products of the fermentation of mackerel contained the alkaloid materials reported by Gautier and Selmi; they were oily colorless liquids, which turned litmus blue and neutralized strong acids. Their reactions with HCl, nitric acid, potassium ferricyanide, and ferric salts, were typical reactions of the ptomaines. They were precipitated by bromine, iodine, phosphomolybdates, etc. They resinified rapidly, their hydrochlorides were neutral and their chloroplatinates crystalline and sparingly soluble (Gautier and Étard, 1882b).

Shortly thereafter Gautier and Étard published a preliminary report describing two alkaloids they had separated from putrefied bodies. Both were oily liquid bases that colored litmus paper strongly blue, attacked tissues in the same manner as KOH, neutralized strong acids, and seemed to absorb CO_2 from air forming crystallizable carbonates (Gautier and Étard, 1882c). Gautier exhibited to the Société de Chimique a sample of one these carbonates. It was a bitter, very caustic and colorless substance boiling at 210 ^{0}C and relative density 1.0296 at 0 ^{0}C. Its hydrochloride crystallized as fine needles, was very bitter and very soluble. The other alkaloid boiled at a higher temperature while decomposing into ammonia and other substances smelling like phenol. Both alkaloids seemed to oxidize and polymerize rapidly, turning into a brown substance, partially soluble in HCl (Gautier and Étard, 1882c).

The main products of putrefaction were separated by the following procedure (Gautier and Étard, 1883a): The solid and liquid materials were distilled under vacuum at low temperature. The passing liquid (A) was found to contain a large amount of ammonium carbonate, phenol, skatole, trimethylamine, and volatile fatty acids. The residue was first extracted with ether and then by alcohol. The ethereal extract (B) separated the ptomaines as well as an abundant quantity of a fatty acid. The alcoholic extract (C) separated the remaining fatty acids and the acid and neutral nitrogenated components. The insoluble residue was boiled with HCl and then extracted with alcohol to yield a new alcoholic solution (D). Each of these portions (A, B, C, D) was further separated and found to contain: (A) a hydrocollidine; (B) an amidostearic acid of formula $C_{18}H_{35}(NH_2)O_2$, insoluble in water, very soluble in hot alcohol, and melting at 63 ^{0}C; (C), and most of the leucines and leucoproteins. The main component of fish meat was the hydrate of a glycoprotein of formula $C_{11}H_{26}N_2O_6$, a white substance, very soluble in water, and crystallizing as white rhomboids (Gautier and Étard, 1883a).

Gautier and Étard gave a detailed description of the procedure used to separate all the acids produced during the action of bacteria during the putrefaction of albuminoidal substances. These acids were classified as fatty, acrylic, lactic, and oxalic series, and nitrogenated acids (Gautier and Étard, 1883b).

Chlorophyll

Étard studied in detail the substances that accompany the chlorophyll present in vegetables (Étard, 1892abd, 1894a, 1895, 1896b, 1897a; a summary appears in Étard, 1894b, 1898). He remarked that the pertinent analytical methods were especial due to the instability of biological materials, which were easily affected by ordinary reagents and could only be treated with neutral solvents or certain diluted chemicals. The correct procedure should start with green plants dried in the shadow, pulverized, and followed by extraction with carbon disulfide. The pertinent dry extract would leave a solid residue, which should then be

extracted with alcohol and ether. These two solvents were able to extract a multitude of substances belonging to all chemical functions. The alcoholic extract should be diluted with carbon disulfide, capable of dissolving a limited number of substances (hydrocarbons, oils, and fatty acids), which were hardly soluble in the first extract. The alcoholic extract, evaporated to dryness, left a thick elastic residue (Étard, 1892d, 1894a, 1898).

According to Étard, it was recommendable to use the following systematic procedure with the carbon disulfide (A) and alcoholic extracts (B). *Group* A1: Upon evaporation, it leaves a green mass, which extracted and crystallized from benzene and ethyl acetate and decolorized with animal carbon, always abandons white crystallizable materials belonging to the chemical series of solid hydrocarbons, alcohols, glycols, and higher polyalcohols. *Group* A2: The alcoholic mother liquors of the previous stage are distilled and then treated with a 2% solution of KOH exempt of NaOH and an excess of ether. This step separates the alkaloids, glycols, and chlorophylls. *Group* A3: The alkaline solutions of the previous step are acidulated in the presence of ether. This step separates the saturated and unsaturated fatty materials, particularly palmitic and oleic acids. *Group* B4: The extract is exhausted with cold alcohol and the tincture separated and re-extracted with ether. This step separates the glucoses, tannins, and salts. *Group* B5: The previous extract is evaporated leaving chlorophyllic material that can be purified. *Group* B6: The alcoholic tinctures of step B4 are distilled and the residue extracted with cold ether, to separate a large quantity of chlorophylls. *Group* B7: the residual of the ether extraction of B6 contains colorless nitrogenated substances, closely related to the chlorophylls (Étard, 1892d, 1894a, 1898).

The first study was devoted to the chlorophyll present in the pericarp of white raisins (Étard, 1892a). Microscopic examination of thin cuts of green leaves, or of this pericarp treated with carbon disulfide, showed that the protoplasm aqueous solutions had not been affected while the green corpuscles had been partially cleaned by the solvent. The extract was loaded with chlorophyll and part of the principles that accompanied it. Due to solubility reasons it did not contain traces of sugars, gums, salts, and of the acids contained in the cellular fluid and in the water, which is the exchange vehicle of the colorless protoplasm. Étard remarked that in order to carry on a proper analysis it was necessary to start from a very large amount of raw vegetable matter (100 kg), which produced of only 5-10 kg of dry matter, containing only 0.3 to 1.0 kg of extract. Treatment of the CS_2 extract with KOH, followed by dilution with water, left the acid and the chlorophyll in the saponified palmitic acid solution. Filtration separated a white substance soluble in ether, crystallizing as long needles melting at 304 ^{0}C and being dextrorotatory with $\alpha_D = +60.8^0$ in ethereal solution, and of formula $C_{26}H_{39}(OH)_3,H_2O$. Étard named this polyalcohol *œnocarpol* and remarked that it had an exceptional stability because, the product that distilled at 405 ^{0}C, after the water, maintained its rotatory power (Étard, 1892a).

The next paper studied the green matter of leaves after the fruit had matured, in three different types of plants: grapevine (*Chasselas*), alfalfa (*Medicago sativa*), and English mandrake (*Briona dioica*) (Étard, 1892b). By an appropriate procedure Étard extracted from grapevine a waxy crystalline matter, melting at 74 ^{0}C, distillable at 300 ^{0}C, and of formula $C_{17}H_{34}O$, which he named *vitol*, and a second one, *vitoglycol* of formula $C_{22}H_{42}(OH)_3$. These new materials were accompanied by palmitic acid. By a similar procedure, the leaves of alfalfa yielded a crystalline waxy substance, of formula $C_{20}H_{41}(OH)$, melting at 80 ^{0}C, boiling at 395 ^{0}C without alteration, and also passing through the digestive system of a dog without alteration. The mandrake extract yielded a white substance crystallizing as flakes like boric acid, insoluble in alcohol, melting at 69 ^{0}C, boiling at 400 ^{0}C without alteration, of formula $C_{20}H_{42}$ (a saturated hydrocarbon), which Étard named *bryonane*. Étard remarked that all these highly stable compounds that he had found probably protected the leaves from the prolonged

action of water and the dissolving liquids secreted by microorganisms. These were the materials normally called *leaf wax* (Étard, 1892b).

In the following publication Étard considered the question if there was only one chlorophyll, common to all green species, or there were several different varieties. For this purpose he selected again the leaves of alfalfa (*Medicago sativa*) (Étard, 1894a). From 480 kg of raw leaves he obtained 50 kg of dry material, which he extracted several times with carbon disulfide and then with alcohol of 95%. The alcoholic extract was dried by distillation, leaving 1.350 kg of a greasy residue containing all the chlorophyll present in the original vegetable. This green material was completely soluble in carbon disulfide. The fact that it was not dissolved in the first extraction with the latter solvent indicated that the chlorophyll was retained as a combination. From the final residue Étard was able to separate four different and perfectly defined chlorophylls, using the procedures described before. The solid residue was extracted with cold alcohol, leaving behind the medicagol. The new alcoholic liquor was extracted with ether and then with pentane. The resulting chlorophyll was soft and amorphous, soluble in carbon disulfide, insoluble in water, and devoid of tannins, glucoses, and vegetable acids. The chlorophyll insoluble in pentane was denser than water, insoluble in concentrated KOH, soluble in diluted alkalis, and formula $C_{28}H_{45}NO_4$. Étard named this chlorophyll *medicagophyll-α* (Étard, 1894a).

The second chlorophyll was separated from the alcoholic extract mentioned above. It was also insoluble in pentane, was denser and insoluble in water, and had formula $C_{42}H_{63}NO_{14}$. Étard named it *medicagophyll-β* (Étard, 1895). Étard mentioned that chlorophylls dissolved in fused KOH or in sulfuric acid monohydrate transformed into a brown coloring matter, which precipitated from these media and re-dissolved, maintained the red fluorescence and the absorption spectrum considered characteristic of chlorophyll. From these facts it could be concluded that the green matter present in leaves contained a fundamental nucleus, very stable, that carried the function of optic absorption connected to the biological work. To this nucleus different chemical groups could be attached, in a more or less permanent manner, giving place to chlorophylls that differed in their composition, molecular mass, solubility, isomerism, etc. and the role that played in living plants. The synthesis action of a plant led to the simultaneous formation of fatty substances insoluble in water and substances clearly soluble, through the intervention of green absorbing bodies, Étard wrote that it was natural to think that one chlorophyll would be insufficient to carry all this chores, and that his results justified this assumption. Certain chlorophylls, soluble in pentane, were probably the instruments for manufacturing the essences and oils. Others, insoluble in hydrocarbons, miscible with water and rich in oxygen, would split and generate the carbohydrates, the tannins, and extracts (Étard, 1895). Étard said nothing regarding the other two varieties of chlorophyll he claimed to have discovered.

Étard summarized his work about chlorophyll in a book published in 1906 (Étard, 1906).

Albumins-leucine

Étard wrote that Henri Braconnot's (1780-1855)) study of the effect of sulfuric acid on glue represented our first knowledge of the constitution of albumins and the discovery of glycocoll (glycine) and leucine (Braconnot, 1820, Étard, 1900). Afterwards, Paul Schützenberger (1829-1897) reported the hydrolysis of albumins by means of barium hydroxide and the presence of acid amines and of more complicated oxygenated bodies, the glycoproteins. The latter were the basic product of the split of albumins by alkalis under pressure (Schützenberger, 1879). According to Étard, the action of alkalis at temperatures of 160^0-$200\ ^0C$ could very well exceed their dehydrating power and generate secondarily CO_2 and acetic and oxalic acid. The presence of these acids was no proof that an oxamide, urea, or purine, was present in albumins, because they also appeared in the alkaline oxidation of saccharides.

Étard chose another road for disintegrating albumins: decomposition of the albumins and glycoproteins by means of sulfuric acid. Boiling 1 kg of dry supraspinatus muscle of beef with sulfuric acid of 20%, he obtained about 1.65 kg of sugar semi-cristalloid matter (like honey), colorless and highly sweet. From an aqueous solution of this material he was able to separate albumin, having composition by weight, 52.8% carbon, 7.1% hydrogen. 16.5% nitrogen, and 23.6% oxygen, and leucine of composition 54.9% carbon, 9.9% hydrogen. 10.7% nitrogen, and 24.5% oxygen. This first result indicated that the tissue fiber, represented by $C_{48}H_{78}N_{13}O_{16}$, fixed 10 moles of water losing only ammonia, as per the following equation (Étard, 1900):

$$C_{48}H_{78}N_{13}O_{16} + 10H_2O = C_{48}H_{98}N_{10}O_{25} + 3NH_3$$

No doubt that the actual proteinic materials were more complicated and it was necessary to increase the molecular mass substantialy to include other elements important to thw cell, such as sulfur and metals.

In another experiment, the aqueous solution mentioned above was distilled with chromic acid producing CO_2, an aqueous distillate containg HCN and no fatty acids, and a residue of ammonium sulfate and oxalic acid. According to Étard, this result indicated that the hydrocarbon chains of the molecule were intercalated in many places by groups $-NH_2$ and OH. Thus, leucine would be eventually transformed into valeric and butyric derivatives. Besides, it was necessary that the nitrogen be present in a β or γ position. The products of the ligament were very white and showed no optical activity, meaning a cancellation of the rotatory power of the leucine ($+17^0$). Dry distillation of the sugar components of the ligament showed that they did not behave like the leucines, they yielded only about 3% of amylamines or pyridines. According to Étard, the hydrolysis of fibrous tissue with sulfuric acid behaved in the same manner as the hydrolysis of a polysaccharide, without yielding significant amounts of leucine. His results did not indicate that glycoproteins were essentially formed of (Étard, 1900):

$$C_nH_{2n+1}NO_2 + C_nH_{2n-1}NO_2 = C_{2n}H_{4n}N_2O_4$$

In a following paper about the chemical nature of tissues Étard wrote that the protoplasm was represented by an albumin, always containing on the average 16% of nitrogen (Étard 1901a). For him this fact showed that albumins, always derived from protoplasmic life, should be actually named *protoplasmids*, in order to put aside the idea of egg albumin. These protoplasmids were probably very numerous and nothing indicated that they were identical in tissues that exerted the same function, for example, that the muscular or nervous protoplasm had the same composition in all the animal series or in all the mamals. To Étard the protoplasmids were amidated saccarides susceptible of transforming by simple hydrolysis. He considered his hydrolytic procedure with sulfuric acid (described above) to be the best tool to determine the composition of these substances (Étard, 1901ab).

He conducted his first experimens using decalcified bone tissue. He took 20 kg of this raw material and boiled them with 30 kg of sulfuric acid of 40% during 48 hours, without noticing any apparent phenomena. The hydrolytic process carried in contact with air produced a black liquor. The organo-sulfuric sulfuric acids were then diluted with water and neutralized with calcium carbonate, followed by filtration. The filtrate was evaporated in a water bath and yielded a residue containing, among other things, the glycine and leucine of the bones. After some time, this residue turned viscous and looked like honey, riddled with small crystals of glycine, and a bit fluorescent. The crystals were dissolved in a little of water, recrystallized, dried, and washed with boiling methanol. The residue was rich in calcium and

glycine and little soluble in concentrated alcohol. Étard used, afterwards, the mother liquors to study the glycine. According to Étard, the amido acids present were cemented by residues of oxalic acid. The methanol washes, left alone, deposited after some time highly impure leucine. These were redisolved in boiling methanol, recrystallized, dissolved in boiling water, and then crystallized again. Étard wrote that the resulting leucine crystallized as small pearly flakes, levorotatory, and having molecular mass 123. Étard wrote that his experiments on the hydrolysis of decalcified bones showed that raw material separated into three groups of matter: (a) glycine, leucine, and at small amount of tyrosine; (b) a sirupy material very soluble in concentrated methanol; and (c) a material competely insoluble in concentrated methanol. The non-crystallizable matter was diluted with water and then saturated with an excess of barium hydroxide to eliminate the sulfuric acid. After filtration, the excess of barium hydroxide was eliminated with CO_2, which also precipitated any calcium left. The barytic syrup was then extracted twice with concentrated methanol and then evaporated to dryness to a white hard crystalline powder. The matter completely soluble in methanol was very deliquescent, and showed not reaction with the reagents for albumins and alkaloids. Elemental analysis indicated that it contained, by weight, 24.5% carbon, 4.0% hydrogen, 7.4% nitrogen, 34.5% barium, and 29.4% oxygen. The large amount of barium was foreign to the true substance, it derived from the preparation method: The glycine, leucine, and tyrosine were separated by means of boiling methanol. He could not determine if it was a pure substance or a mixture of two derivatives of the same group or isomers (Étard 1901ab).

In a following publication Étard reported that he had found his leucine to be contamined by glutamic acid. In previous work he had learned that leucine hydrochloride was very soluble in water while glutamic acid hydrochloride was highly insoluble. He used this procedure to separate the glutamic acid contaminating in his leucine (Étard, 1901c).

In another work Étard described in detail the construction and operation of the reactor he used to carry on the acid hydrolysis of protoplasmides in large scale (Étard, 1903).

Most of authors believed that common leucine was a normal α-aminocaproic acid, CH_3-$(CH_2)_3$-$CH(NH_2)$-COOH, although others thought that leucine derived from valeral, according to the formula $(CH_3)_2$-CH-CH_2- $CH(NH_2)$ -COOH. Étard and Antony Vila did no believe that this was possible because there were 31 possible leucines and the name valeral inappropriate because 3 isomers of this body were possible (Étard and Vila, 1902). G. Bémont had recently shown that the oxidation of amyl alcohol produced by fermentation and boiling at 131 ^{0}C, yielded an optically active valeric acid boiling at 175 ^{0}C and resembling methylethylacetic acid (Bémont, 1901). According to Étard and Vila, the *active* valeral prepared from the alcohol of Bémont, and distilling at 91^0-92 ^{0}C, could not be normal or dimethylated, but should correspond to the formula CH_3-CH_2-$CH(CH_3)$-COOH. For this reason they used Bémont alcohol to prepare a synthetic leucine in three steps: (1) preparation of an hydrated valeral-ammonium of formula C_5H10O,NH_3 + 8Aq, by reacting the valeral with aqueous ammonia of relative density 0.950 during 24 hours. This material appeared as white, optically active crystals; (2) treating the latter with the theoretical amount of HCN transformed it into an oily material without optical activity, boiling at 115^0-120 ^{0}C at pressure 3-4 mmHg, and having formula $C_{12}H_{18}N_2$, and (3) hydrolysis of the latter with sulfuric acid transformed it into a solid material. Dissolution of this solid, followed by spontaneous evaporation, precipitated acicular crystals of a sulfate containing, by weight, 44.68% carbon, 7.90% hydrogen, 16.48% nitrogen, and 31.9-32.0% SO_4. This salt was very bitter although it should yield a sweet leucine. Hydrolysis with HCl or sulfuric acid liberated an artificial leucine, different from the biological leucine, and containing two assymetric carbons (Étard and Vila, 1902).

After the work of Gautier and Étard and Selmi on the production of poisonous matters (Gautier and Étard, 1882a; Selmi, 1870) by putrefaction, many other papers were published

trying to establish the chemical nature and properties of this group of substances. Several alkaloids were signaled, such as methylamines, collidine, muscarine, etc. Nevertheless, not much was done to identify the most immediate susbtances connected with the decompositon of tissues (Étard and Vila, 1903). Gautier and Étard determined the boiling point, density and formula of the first putrefaction body, $C_8H_{13}N$. Sometime afterwards, Ludwig Brieger (1849-1919) isolated another base, $C_5H_{16}N_2$, which he named *cadaverine* (Brieger, 1885). Ladenburg (1842-1911) then proved that cadaverine was pentamethylene diamine (Landenburg, 1886)***.

Étard and Vila mentioned that during their work on the hydrolysis of tissues they had separated hundreds of grams of a benzoyl derivative (musculamine benzoyl), which afterwards Swigel Posternak (1871-1932) considered to be a benzoyl derivative of cadaverine (Posternak, 1902). This led Étard and Vila to repeat their analysis and confirm the finding of Posternak (Étard and Vila, 1903). They separated this compound by hydrolyzing at 100 ^{0}C ground and boiled calf muscles, with sulfuric acid of 15%. The acid was then neutralized with ammonia and the resulting precipitate of ammonium chloride eliminated by filtration. The filtrate was made alkaline with barium hydrate and then mixed with benzoyl chloride. The precipitate of benzoyl cadaverine was dissolved in boiling KOH. On cooling, the resulting solution deposited pure benzoyl cadaverine as crystalline needles. According to Étard and Vila, the large amount of this compound present in the splitting of corrupt muscles explained the many accidents that these muscles could cause when eaten. According to Brieger, benzoyl cadaverine had necrotic properties and accompanied the serious injuries of the intestine caused by cholera infections (Étard and Vila, 1903). Étard and Vila also discussed the hydrolysis of protoplasmides under the action of acids (Étard and Vila, 1907, 1908a).

Figure 1*:* Alexandre-Léon Étard (1852-1919***).***

*** Ladenburg's constitution is the one accepted today: 1,5-pentanediamine or pentamethylene diamine, formula C5H14N2, NH2(CH2)5NH2.

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