1 General Aspects

Acetylsalicylic Acid, Second Edition. Karsten Schrör.

1
General Aspects

1.1
History

1.1.1
From Willow Bark to Salicylic Acid

1.1.1.1 Anti-Inflammatory and Analgesic Effects of Willow Bark and Leaves

Medical Effects of Willow Bark Treatment of maladies by plants or extracts thereof is as old as the history of mankind. This is also true for fever and pain, two particularly frequent and inconvenient symptoms of acute illnesses but also typical for osteoarthritis and rheumatism, two examples of chronic painful diseases. Rheumatism was already known in old Egypt as seen from cartilage alterations in Egyptian mummies. The Egyptians were also aware of the pain-relieving effects of potions made from myrtle and willow leaves. Clay tablets from the Sumerian period also contained information about the use of willow leaves as medicines. *Hippocrates* recommended leaves of the willow tree for medical purposes about 400 BC. *Pliny* (compilations) and *Dioscurides* (*Materia Medica*) also recommended decocts of willow leaves or ash from willow bark for treatment of sciatica (lumbago) and gout at about 100 AC. Outside Europe, it were the Nama (Hottentots) in Southern Africa who had "for a long time" used tea made from bark of willow trees for treatment of rheumatic diseases (cited after Ref. [1]). This comment was made by Dr. Ensor from Cape Town (South Africa) in his reply to a publication of Dr. MacLagan in 1876 [2] describing for the first time positive experience with salicylates at 2 g/day for treatment of rheumatism.

The First Published Clinical Trial The first known public communication on the medical use of willow bark extracts in modern times came from Reverend *Edward Stone* [3] from Chipping Norton (Oxfordshire, England). He treated some 50 cases of "aigues, fever, and intermitting disorders" with a redissolved powdered dry bark preparation of willow tree. The doses were about "20 gr(ains) [≈1.3 g] to a dram of water every 4 hours." On June 2, 1763, he wrote a letter to the Earl of Macclesfield, the then President of the Royal Society in London, entitled "An account of the success of the bark of the willow in the cure of aigues." In this letter, he summarized his opinion about this treatment as follows:

> *. . . As this tree delights in moist or wet soil where agues chiefly abound, the general maxim, that many natural maladies carry their cure along with them or that their remedies lie not far from their causes, was so very apposite to this particular case, that I could not help applying it; and this might be the intention of providence here, I must own had some little weight with me . . . .*

After claiming to have obtained good results, he concluded:

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. . . I have no other motives for publishing this valuable specific than that it may have a fair and full trial in all its variety of circumstances and situations, and that the world may reap the benefits accruing from it.

1.1.1.2 Salicylates as the Active Ingredients of Willow Bark and Other Natural Sources

Detection and Preparation of Salicin from Willow Bark In 1828, the German pharmacist *Johann Andreas Buchner* was the first to prepare a yellowish mash with bitter taste from boiled willow bark, which he named Salicin, after the Latin word for willow *(salix).* He considered salicin as the active antipyretic ingredient of willow bark and recommended its use for treatment of fever. A similar conclusion had earlier been reached by the Italians *Brugnatelli* and *Fontana* in 1826 using a less purified preparation of willow bark. They also considered salicin as the active principal component of willow bark (cited after Ref. [4]). In 1830, the Frenchman Henry *Leroux* was the first to obtain salicin in crystalline form. Only 3 years later, in 1833, the pharmacist *Merck* in Darmstadt (Germany) announced highly purified salicin from willow bark for use as an antipyretic for half of the price of quinine (cited after Ref. [5]) – at that time a really attractive offer.

Salicin from Natural Sources as Starting Material to Make Salicylic Acid Salicin is not only the active antipyretic ingredient of willow bark but also the reason for its strong bitter taste and the irritation of stomach mucosa. Both limited its practical use. Salicin hydrolyzes in aqueous media to glucose and salicylic alcohol (saligenin). Saligenin has no bitter taste and can be easily oxidized to salicylic acid. *Raffaele Piria*, an Italian, was the first to successfully synthesize salicylic acid (acide salicique ou salicylique) from salicin in 1839 and also correctly determined the empirical formula $C_7H_6O_3$. This led to the possibility of replacing the poorly palatable salicin by salicylic acid, for example, as a good water-soluble sodium salt. This became practically relevant after new and abundant natural sources for salicylates were detected. These included wintergreen oil obtained from the American Evergreen (*Gaultheria procumbens*) and spireic acid (acidum salicylicum) from the American teaberry (*Spiraea ulmaria*). Gaultheria oil (wintergreen oil) consists of about 99% of methyl salicylate from which free salicylic acid can easily be obtained. However, production of salicylates by plants is also an important defence mechanism in itself.

*Efficient communication between the pest-colonized and noncolonized plants is vital for timely manifestation of defenses that restrict systemic spread of pests. Airborne signals are involved in these processes. Methyl salicylate is a volatile compound that is made by a number of plants and is suggested to act as a mobile airborne signal in plant defence by activation of systemic acquired resistance. This confers enhanced resistance against a broad spectrum of pathogens (*Section 2.2.2*)* [6].

1.1.1.3 Chemical Synthesis of Salicylic Acid

The Kolbe–Schmitt Synthesis The modern pharmaceutical history of salicylates and its derivatives starts with the chemical synthesis of the compound. In 1859, *Hermann Kolbe*, a German and Professor of Chemistry in Marburg, produced the first fully synthetic salicylic acid from the already known decomposition products phenol and carbonic acid, that is, sodium phenolate and carbon dioxide. Kolbe then stimulated his assistant *Rudolf Wilhelm Schmitt* to further improve the technology, eventually resulting in doubling of the salicylic acid yield. Schmitt also elucidated the reaction kinetics. This base-promoted carboxylation of phenols under high pressure allowing the synthesis of salicylic acid derivatives is known since then as the "Kolbe–Schmitt reaction." *Friedrich von Heyden*, a student of Schmitt, was introduced to Kolbe who encouraged him to develop a procedure to make the compound on an industrial scale. Von Heyden was the first to receive a patent for this procedure. The development of an appropriate technology to synthesize large amounts of

salicylate, independent of the limited availability of natural sources with varying contents and seasonal variations of the active ingredient, opened the door for its broader practical use and thus caused a massive drop in price: The price of 100 g of salicylic acid prepared from salicin from natural sources (gaultheria oil) dropped from 10 to 1 Taler/100 g (Dollar = American for Taler) for the chemical product made through Kolbe's synthesis (cited after Ref. [7]).

Von Heyden started the large-scale production of salicylic acid in the kitchen of his mansion, the "Villa Adolpha" in Dresden (Saxony). In 1874, the site was moved to Radebeul, a suburb west to Dresden, where he founded the factory "Salizylsäurefabrik Dr. von Heyden." This plant was extremely effective: After making 4 tons of salicylic acid in the first year, the annual production was increased to 25 tons only 4 years later and continued to grow steadily. Kolbe and von Heyden received patents for the synthesis of salicylate in many European countries and the United States [8]. Interestingly, after solving some legal issues, von Heyden's plant also produced the salicylic acid that was later used by Bayer to make aspirin [9].

Practical Use of Salicylate After salicylate as a cheap chemical became available on an industrial scale, that is, in essentially unlimited amounts, the compound was tested for new practical applications. For example, salicylic acid was soon found to have antiseptic properties that could be used to preserve milk and meat. The compound was also recommended as an alternative to phenol (carbolic acid), which, was the antiseptic of choice in surgery those days. The antipyretic action of salicylate was for a time also attributed to its antiseptic activity, until it was shown that the sodium salt with little antiseptic properties was an equally effective antipyretic (cited after Ref. [1]). Importantly, salicylic acid was also studied as a potential drug in a large variety of diseases and thus became the first synthetic drug ever developed. In 1875, *Ebstein and Müller* [10] detected the blood sugar-lowering action of the compound. Shortly thereafter, the uricosuric action of salicylate was described. Thus, salicylates appeared to be useful for treatment of diabetes and gout.

Salicylic Acid as an Anti-Inflammatory Antirheumatic Agent Of the several discoveries regarding medical applications of salicylates, the most significant was the finding that synthetic salicylates were potent anti-inflammatory analgesics and extremely useful for treatment of rheumatic diseases. *Franz Stricker* from Berlin was the first to publish that sodium salicylate was not only an antipyretic remedy but also as an effective drug for treatment of rheumatic bone and joint diseases [11]. He was the first to clinically introduce salicylate in 1876 as an analgesic antirheumatic drug at the Charité in Berlin [12]. Two months later, Scottish physician *Thomas J. MacLagan* [13] published the first of a series of articles showing that administration of salicylate to patients with rheumatic fever resulted in the rapid disappearance of fever and pain. Similar results were reported by the Frenchman *Germain Sée* 1 year later [14]. These three studies marked the beginning of the systematic therapeutic use of salicylates as analgesic anti-inflammatory drugs in medicine.

Summary

Extracts or other preparations from willow bark or leaves were used in ancient times as household remedies for the treatment of fever, inflammation, and pain. These ancient uses have been rediscovered only in the eighteenth century: In 1763, the first communication on successful use by Reverend Edward Stone of an aqueous extract of powdered willow bark in the treatment of "aigue and feverish diseases" was published in the United Kingdom.

Search for the active ingredient of willow bark initially resulted in the detection of salicin, from which salicylate as the active fraction could easily be prepared. Further rich natural sources of salicylates were found, among them being the American evergreen (*Gaultheria procumbens)* and spireic acid (acidum salicylicum) from the American teaberry (*S. ulmaria*).

Kolbe was the first to succeed in making fully synthetic salicylate from sodium phenolate and carbon dioxide in 1859, a procedure later improved by Schmitt. Some further improvements on procedure by von Heyden eventually resulted in the foundation of the plant "Salicylsäurefabrik von Heyden" in 1874 that produced salicylic acid in large scale. This new compound was not only accepted for wide practical use but also became the first entirely synthetic drug worldwide; it was introduced in the clinics as an analgesic antirheumatic by Stricker in Berlin 1876.

References

1 Gross, M. and Greenberg, L.A. (1948) *The Salicylates: A Critical Bibliographic Review*, Hillhouse Press, New Haven, CT.

2 MacLagan, T. (1876) The treatment of rheumatism by salicin and salicylic acid. *BMJ*, **1** (803), 627.

3 Stone, E. (1763) An account of the success of the bark of the willow in the cure of agues. *Philos. Trans. R. Soc. Lond.*, **53**, 195–200.

4 Sharp, G. (1915) The history of the salicylic compounds and of salicin. *Pharm. J.*, **94**, 857.

5 Horsch, W. (1979) Die Salicylate. *Pharmazie*, **34** (9), 585–604.

6 Shah, J. (2009) Plants under attack: systemic signals in defence. *Curr. Opin. Plant. Biol.*, **12** (4), 459–464.

7 Bekemeier, H. (1977) On the history of the salicylic acid. *Wiss. Beitr. Martin Luther Univ. Halle Wittenberg*, **42** (R34), 6–13.

8 Reschetilowski, W., Remane, H., and Schuhmann, A. (2012) *Historische Stätten der Chemie: Ehemalige Salicylsäurefabrik und spätere Chemische Fabrik Dr. F. von Heyden Radebeul*, Gesellschaft Deutscher Chemiker.

9 Schreiner, C. (1997) *100 Years of Aspirin. The Future Has Just Begun*, Bayer AG.

10 Ebstein, W. and Müller, J. (1875) Weitere Mittheilungen über die Behandlung des Diabetes mellitus mit Carbolsäure nebst Bemerkungen über die Anwendung von Salicylsäure bei dieser Krankheit. *Berlin Klin. Wochenschr.*, **12**, 53–56.

11 Stricker, S. (1876) Über die Resultate der Behandlung der Polyarthritis rheumatica mit Salicylsäure. *Berlin Klin. Wochenschr.*, **13**, 1–2, 15–16, 99–103.

12 Hangarter, W. (1974) Herkommen, Geschichte, Anwendung und weitere Entwicklung der Salizylsäure, in *Die Salizylsäure und ihre Abkömmlinge* (ed. W. Hangarter), F.K. Schattauer, Stuttgart, pp. 3–11.

13 MacLagan, T. (1876) The treatment of rheumatism by salicin and salicylic acid. *British Med. J.*, **1** (803), 627.

14 Sée, G. (1877) Études sur l'acide salicyliqué et les salicylates; traitement du rhumatisme aigu et chronique, de la goutte, et de diverses affections du système nerveux sensitif par les salicylates. *Bull. Acad. Nat. Med. (Paris)*, **6**, 689–706, 717–754.

1.1.2 Synthesis of Acetylated Salicylic Acid and First Medical Use

1.1.2.1 The Invention of Acetylated Salicylic Acid

Despite the undisputed benefits of sodium salicylate in the treatment of pain, fever, and inflammatory disorders, there were several problems with the practical handling of the compound. These included an unpleasant sweetish taste and, in particular, irritations of the stomach, often associated with nausea and vomiting. Another disturbing side effect was a hearing disorder called tinnitus. These side effects occurred quite frequently at high doses of several grams of salicylate per day, which had to be taken regularly by patients suffering from chronic (rheumatic) pain. Thus, after an effective technology to generate large amounts of entirely synthetic salicylate became available, several efforts were made to improve the pharmacological properties of the compound by appropriate chemical modifications, eventually resulting in increased efficacy as well as improved gastric tolerability when the substance was made more palatable. Acetylation was a favored chemical method at the time to reach this goal. Several researchers addressed this issue by acetylating salicylic acid with different results [1–3] until chemists of the firm of "Farbenfabriken Bayer" in Elberfeld, today part of Wuppertal (Germany), succeeded in synthesizing acetylated salicylic acid from salicylic acid and acetic anhydride in a chemically pure and stable form.

The History of Bayer Aspirin Three persons at Bayer were intimately involved in this development. All three were chemists and all of them were of the same age group – born in the 1960s, when the knowledge in organic chemistry had just started to explode. The first to be named was *Carl Duisberg* (Figure 1.1.2-1). After completing his dissertation in Jena with a study on acetoacetate esters (!), he decided to pursue his career in the chemical industry. In 1883 he joined the Bayer company and became head of the research only 5 years later [4]. *Arthur Eichengrün* (Figure 1.1.2-1) joined the Bayer Company in 1895 and became head of the Pharmaceutical Research Department that was newly founded by Duisberg [5]. According to a report, written by Eichengrün 50 years later [6], it was his idea to acetylate salicylate in order to make it more palatable and also avoid the unpleasant irritation of the stomach. As mentioned above, the concept of acetylation of drugs to improve their efficacy was not new at the time. It had already been successfully used in making phenacetin, a powerful analgesic, by the Bayer Company. Phenacetin was synthesized via acetaminophen from *p*-nitrophenol, a waste product of Bayer's dye fabrication [4]. This positive experience probably stimulated the company to make a more widespread application of acetylation procedures to other chemicals and drugs. This included guaiacol, cinchonine, morphine – and salicylic acid. *Felix Hoffmann* (Figure 1.1.2-1) was the chemist

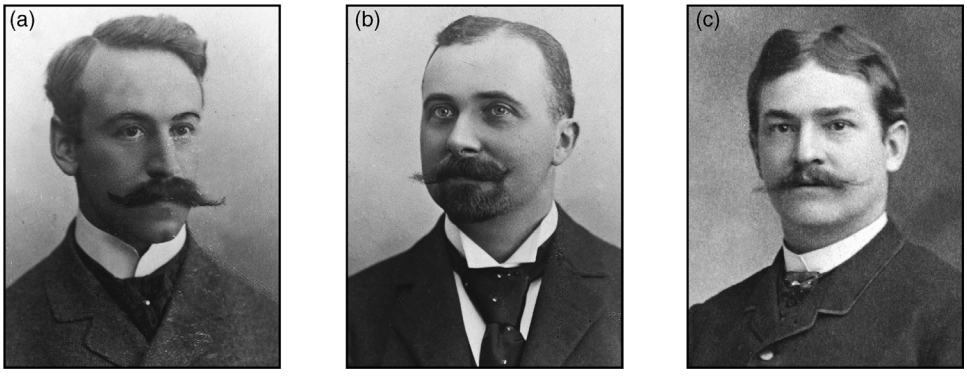


Figure 1.1.2-1 (a) Arthur Eichengrün (1867–1949). (b) Felix Hoffmann (1868–1946). (c) Carl Duisberg (1861–1935). (With kind permission from Bayer.)

working on this issue "on Eichengrün's advice" [6]. He was the first person to develop a technology to produce chemically pure and stable acetylsalicylic acid from salicylic acid and acetic anhydride. According to a handwritten note in his laboratory diary, this success was achieved on August 10, 1897, (Figure 1.1.2-2). Later he wrote:

> *. . . When salicylic acid (100.0 parts) is heated with acetic anhydride (150.0 parts) for 3 hours under reflux, the salicylic acid is quantitatively acetylated . . . . By its physical properties, e.g. its sour taste without being corrosive, the acetylsalicylic acid differs favorably from salicylic acid, and is now being tested in this respect for its usefulness*

Another person at Bayer should also be mentioned in this context: *Heinrich Dreser*, the then head of the Department of Pharmacology. Dreser was not interested in this kind of research and initially did not believe in any clinically useful properties of the new compound ("the compound is of no value"). However, in his later description of the pharmacology of aspirin, he acknowledged the better taste and less gastric irritation [7]. Initially, he was also not informed by the pharmacists about its successful clinical testing, although according to his contract with the company, the pharmacists should have had reported this finding to him before undertaking further activities [2]. Thus, he was probably not amused to learn that without his knowledge and against his declared intention, the new compound was – even successfully – tested in patients. According to Eichengrün and other sources, he did everything to block the further development of aspirin, while Duisberg emphatically supported the activities of Eichengrün and Hoffmann and, as expected, finally succeeded. The further development and clinical introduction of acetylated salicylate as an antipyretic analgesic, eventually resulting in the worldwide spread of the compound, is his merit.

The new drug received the trade name "aspirin," which is composed from "*a*cetic" and "*spir*eic acid," a former name of *o*-hydroxybenzoic acid (salicylic acid), originally prepared from *S. ulmaria*, one of the richest natural sources of salicylates.

The first description of the pharmacology of aspirin was published in 1899 by Dreser. The names of the two chemists Hoffmann and Eichengrün were not mentioned in this paper. Dreser considered aspirin as a better tolerable prodrug of the active metabolite salicylic acid with the positive pharmacodynamic property not to be cardiotoxic [7]. According to Eichengrün [6], Dreser had nothing to do with the invention. However, it was Dreser who took the financial benefits from the discovery, not Eichengrün and Hoffmann [8]. According to a contract with Bayer, the products invented under the direction of Eichengrün had to be patented in Germany to get a royalty for the inventor from the company [6]. Acetylsalicylic acid was registered on February 1, 1899 under the trade name "Aspirin®" by the Imperial Patent Bureau (Kaiserliches Patentamt) in Berlin and a few weeks later introduced to the market. This was the first time that a drug was dispensed as a product (powder or tablet) made by chemists according to quality standards of their company and not dispensed as a product manufactured as a powder by a pharmacist. This caused long-lasting and intense discussions about the role of pharmacists as the primary controller of drug production [9].

"Aspirin" did not receive recognition as a drug to be patented in Germany or any other European country, except the United Kingdom, where Bayer held a patent until 1905. This patent was declared futile by a British Court in 1905 after a legal action of von Heyden company, the provider of salicylic acid for Bayer. Von Heyden also produced and sold acetylated salicylic acid, but under its chemical name "acetylsalicylic acid" [8].

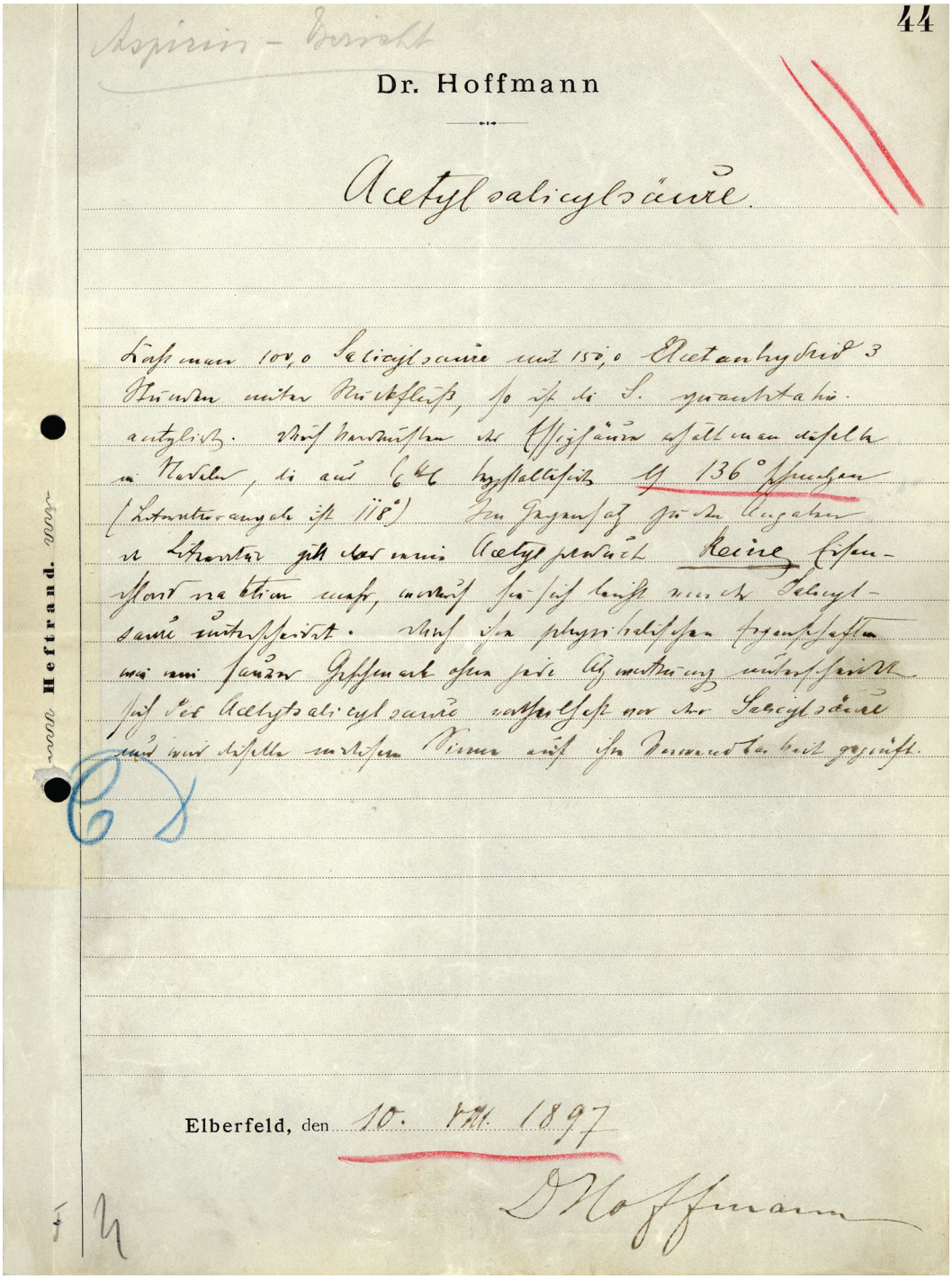


Figure 1.1.2-2 Laboratory record of Dr. Felix Hoffmann from August 10, 1897 containing the first description of successful synthesis of acetylsalicylic acid. (With kind permission from Bayer.)

Bayer marketed the new compound under the Bayer-owned trade name "Aspirin." In the labeling, the product was identified as "monoacetic acid ester of salicylic acid" and advertised as a better tasting replacement for salicylic acid. The aspirin packages did not indicate that aspirin was pure acetylsalicylic acid. Bayer took every effort to keep this trade name as sole property of Bayer. The (numerous) copycats had to use other labeling, mostly they preferred the chemical term "acetylsalicylic acid" while Bayer advertised aspirin as "best replacement for salicylic acid." Doctors (probably) never learned from Bayer's advertising that aspirin was solely a trade name and found it easier to prescribe "Aspirin(um)" [sic!] than "acetylsalicylic acid" [8].

Aspirin was patented 1900 exclusively in the United States (Figure 1.1.2-3).

As a consequence of World War I, in 1917, all patents and trade names of German firms were held enemy property in the United States and, thus, were confiscated [9]. German firms were also no longer allowed to sell their products in the United States [8]. The Bayer assets were auctioned by the US Alien Property Custodian and sold the same year for $5.3 millions to Sterling Drugs, Inc. of New York [10,11]. This company then produced "genuine Bayer Aspirin" for the US market (Section 3.1.1) [12]: It was only in 1994 that the German Bayer AG could buy back the rights of the trademark and the Bayer Cross in the United States (for details, see Ref. [8]).

According to a publication by Sneader and some followers, not Hoffmann but rather Eichengrün should be considered as the true inventor of aspirin [13]. As Eichengrün was Jewish, he could not enjoy the fruits of his remarkable scientific research, including also the invention of several other products in addition to aspirin, such as acetate silk, because of the political reasons during the Nazi regime. Eichengrün was interned in 1944 in a concentration camp and remained there until the end of World War II. Eichengrün in the year of his death (1949) stated in an article, published in the German Scientific Journal *Die Pharmazie*, that it was he and Felix Hoffmann who should be considered as the inventors of aspirin [6].

According to Eichengrün [6], it was Hoffmann who had first worked out the acetylation technology (. . . "welcher [Hoffmann] die Acetylierung ausgearbeitet hatte" . . .), eventually resulting in the synthesis of pure and chemically stable acetylated salicylic acid [14], although, again according to Eichengrün, he did so following "my advices" ("er führte meine [chemischen] Anordnungen aus"). However, the sole, unopposed mention of Hoffmann's name on the US patent application form of 1900 (Figure 1.1.2-3) clearly would not have been possible without the knowledge or even against the will of his two supervisor chemists, Eichengrün and Duisberg. This clearly suggests that both considered Hoffmann's activities in this research as very fundamental, justifying his name as the inventor of aspirin. Because of the complexity of the issue, as already discussed, one should, however, also pay tribute to the significant contributions of Eichengrün and Duisberg in the research and development of aspirin. This will not reduce the outstanding contribution of Hoffmann in this discovery.

Further Attempts to Make Acetylsalicylic Acid At this point, it should be noted that Hoffmann was not the first person who tried to chemically synthesize acetylated salicylic acid. In 1853, *Charles Frédéric Gerhardt*, a Frenchman, from Straßburg (Alsace) described the synthesis of a new compound from acetyl chloride and sodium salicylate, which he named "salicylate acétique" [15].

This publication of Gerhardt was taken by several authors as evidence to ascribe the invention of acetylsalicylic acid to him (e.g., Refs. [3,10,16]. *This is not correct for several reasons. The "acetylsalicylic acid" of Gerhardt, if it was formed at all, solely might have existed as a labile, intermediate raw product of the reaction between acetyl chloride (prepared by him by a suboptimal procedure) and sodium salicylate* [2]. *The chemical structure of "salicylate acétique" was not determined. The physicochemical properties were not those of acetylsalicylic acid but rather those of salicylic acid* [17,18]. *The technical procedure was suboptimal and resulted in simultaneous formation of large amounts of acetic acid anhydride together with acetosalicylic acid anhydride because of an inappropriate processing of the raw product. As a stable end product, Gerhardt only obtained salicylic acid* [19]. *From his experiments, he concluded that acetylated salicylic acid is unstable and in water immediately breaks down to salicylic acid and acetate* [15]. *Both statements are wrong and do not qualify Gerhardt for the claim to have invented the synthesis of acetylsalicylic acid.*

UNITED STATES PATENT OFFICE.

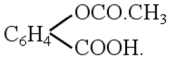
FELIX HOFFMANN OF ELBERFELD GERMANY ASSIGNOR TO THE FARBEN-FABRIKEN OF ELBERFELD COMPANY OF NEW YORK.

ACETYL SALICYLIC ACID.

SPECIFICATION forming part of Letters Patent No. 644,077, dated February 27, 1900.
Application filed August 1, 1898 Serial No. 087,385 (Specimens.)

To all whom it may concern:
Be it known that I, FELIX HOFFMANN, doctor of philosophy, chemist, (assignor to the FARBENFABRIKEN OF ELBERFELD COMPANY, of New York) residing at Elberfeld, Germany, have invented a new and useful Improvement in the Manufacture or Production of Acetyl Salicylic Acid; and I hereby declare the following to be a clear and exact description of my invention.

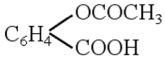
In the *Annalen der Chemie und Pharmacie,* Vol. 150, pages 11 and 12, Kraut has described that he obtained by the action of acetyl chlorid on salicylic acid a body which he thought to be acetyl salicylic acid. I have now found that on heating salicylic acid with acetic anhydride a body is obtained the properties of which are perfectly different from those of the body described by Kraut. According to my researches the body obtained by means of my new process is undoubtedly the real acetyl salicylic acid



Therefore the compound described by Kraut cannot be the real acetyl salicylic acid but is another compound. In the following I point out specifically the principal differences between my new compound and the body described by Kraut.

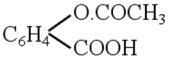
If the Kraut product is boiled even for a long while with water, (according to Kraut's statement,) acetic acid is not produced, while my new body when boiled with water is readily split up, acetic and salicylic acid being produced. The watery solution of the Kraut body shows the same behavior on the addition of a small quantity of ferric chlorid as a watery solution of salicylic acid when mixed with a small quantity of ferric chlorid—that is to say, it assumes a violet color. On the contrary, a watery solution of my new body when mixed with ferric chlorid does not assume a violet color. If a melted test portion of the Kraut body is allowed to cool it begins to solidify (according to Kraut's statement) at from 118° to 118.5° centigrade while a melted test portion of my product solidifies at about 70° centigrade. The melting-points of the two compounds cannot be compared because Kraut does not give the melting-point of his compound. It follows from those details that the two compounds are absolutely different.

In producing my new compound I can proceed as follows, (without limiting myself to the particulars given:) A mixture prepared from fifty parts of salicylic acid and seventy-five parts of acetic anhydride is heated for about two hours at about 150° centigrade in a vessel provided with a reflex condenser. Thus a clear liquid is obtained, from which on cooling a crystalline mass is separated, which is the acetyl salicylic acid. It is freed from the acetic anhydride by pressing and then recrystallized from dry chloroform. The acid is thus obtained in the shape of glittering white needles melting at about 135°centigrade, which are easily soluble in benzene, alcohol, glacial acetic acid, and chloroform, but difficultly soluble in cold water. It has the formula



and exhibits therapeutical properties.
Having now described my invention and in what manner the same is to be performed, what I claim as new, and desire to secure by Letters Patent, is—

As a new article of manufacture the acetyl salicylic acid having the formula:



being when crystallized from dry chloroform in the shape of white glittering needles, easily soluble in benzene, alcohol and glacial acetic acid, difficultly soluble in cold water being split by hot water into acetic acid and salicylic acid, melting at about 135° centigrade substantially as herein before described.

In testimony whereof I have signed my name in the presence of two subscribing witnesses.

FELIX HOFFMANN.

Witnesses:
R. E. JAHN,
OTTO KÖNIG.

Figure 1.1.2-3 The US acetylsalicylic acid patent from February 27, 1900. (With kind permission from Bayer HealthCare.)

In 1859, *Hugo von Gilm*, a pharmacist from Innsbruck (Austria), reported on the synthesis of acetylsalicylic acid [20] as did *Karl Kraut* and his group from Hannover (Prussia) 10 years later [17]. Kraut and his coworkers Schröder and Prinzhorn were also the first to assign the correct structure with the acetyl moiety connected to the phenolic oxygen to the compound. However, these preparations were still impure and contained significant amounts of salicylic acid, as seen from the positive red "Gerhardt-reaction" of salicylate with ferric chloride (Section 2.1.1). In addition, it exhibited physicochemical properties different from acetylsalicylic acid [19,23] (see also comments of Hoffmann in his patent application) (Figure 1.1.2-3). Nevertheless, it was the publication of Kraut that was the reason for decline of patent protection of the Hoffmann synthesis by the German Patent Authorities [21].

Acetylsalicylic Acid: Organic Chemistry versus Pharmacology In contrast to the natural product salicylic acid, *acetyl*salicylic acid could be made only by organic chemistry – although Karl Kraut started his experiments for its synthesis with gaultheria oil as a natural salicylate (salicin) source. During the following 30 years, there were no further attempts to improve the synthetic procedure, although significant progress was made in organic and pharmaceutical chemistry at this time. According to an organic chemist, acetylsalicylic acid was of no particular interest, but solely made to confirm the feasibility of its synthesis. There were also no ideas or concepts about any possible practical application, including its use as a therapeutic. Thus, acetylated salicylic acid probably would have suffered the fate of several hundreds of chemicals before and many thousands thereafter – a product of chemical synthesis, principally easy to make but more difficult in pure and chemically stable form and without any practical value.

Hoffmann and Eichengrün, in contrast, have had combined the available medical knowledge about curative properties of a product from nature with the contemporary organic chemistry with a clear intention to make a new and better therapeutic out of it. These studies would not have been possible without the substantial and continuous support of Carl Duisberg, the then Head of Bayer research. Duisberg later became Chief Executive and Director General at Bayer. His numerous and outstanding efforts inside and outside the Bayer company gained considerable and consistent recognition, in both Germany and abroad [4]. In an obituary in 1935, the London *Times* noted: . . . "his country loses a man who, all things considered, . . . may be regarded as the greatest industrialist the world has yet had . . . ." Therefore, the company had good reason to duly celebrate the 100th anniversary of "his" compound that in the meantime had become the most popular drug in the world [13].

In the context of priorities in science, an interesting comparison between the discovery of aspirin and the discovery of prostacyclin can be made – both also tightly connected with the name of John Vane. Its chemical structure as well as a suggested (later confirmed) enzymatic synthetic pathway was originally described in 1971 by Pace-Asciak & Wolfe. These authors considered the (labile) product as just another prostaglandin – in addition to the dozens of already known compounds. The authors assumed that it was possibly overlooked by earlier investigators because of its low biological activity, tested at the time in bioassay experiments using the rat stomach strip. It also remained uncertain whether the compound was synthesized at all in the intact stomach wall and, if so, was released in biologically active amounts [22].

A completely different approach was followed by the group of John Vane. Their work on prostacyclin started with the discovery of a biological effect – inhibition of platelet aggregation – by an enzymatic product made from prostaglandin endoperoxides by artery walls [23]. *This prostaglandin, originally named as PGX, differed in its biological property from all other known prostaglandins. PGX was later identified as the already known enzymatic product of prostaglandin endoperoxides, described by Pace-Asciak & Wolfe, and was renamed prostacyclin (PGI_2).*

Despite the originality and merits of Pace-Asciak & Wolfe regarding the detection and original description of biosynthetic pathways of natural prostacyclin and its suggested chemical structure, the medical history of

prostacyclin starts with the work of Vane's group who were the first to discover the biological significance of prostacyclin in control of hemostasis.

1.1.2.2 Introduction of Acetylsalicylic Acid into the Clinics

The First Clinical Trials *Kurt Witthauer* [24], the then Senior physician in the (still existent!) city hospital (Diakonie Krankenhaus) in Halle/Saale (Germany), and *Julius Wolgemuth* [25] from Berlin published the first clinical investigations on aspirin in 1899. In his publication, Witthauer first outlined the pharmacological advantages of aspirin over salicylate, that is, its chemical stability in the acidic stomach juice while cleavage of (the active) salicylate only occurs in the alkaline intestinal fluid. Because of this, he would expect a better gastric tolerance of the new compound. Then, he reported on treatment results in about 50 patients suffering from a variety of inflammatory, mostly rheumatic, diseases. They received 4–5 g of aspirin daily obviously without any complaints. Witthauer started his account as follows:

. . . Nowadays, certain courage is necessary to recommend a new drug. Almost every day those are thrown on the market and one has to have an excellent memory to keep all the new names and brands in mind. Many drugs appear, are praised and recommended by the companies and certain authors but after a short time have disappeared without any further comment . . .

The author also did not forget to instruct his readers that he did this study with "no little distrust." Regarding the results, he commented:

. . . the treatment result was at least as good as that of natron [salicylate], sometimes [aspirin] was even effective when natron failed . . .

and added that

. . . the patients were unsatisfied, if it became necessary to interrupt the aspirin treatment because of an insufficient supply . . .

Witthauer concluded:

. . . According to my positive results, the company is now prepared – after waiting for quite a time – to introduce the new compound on the market. I sincerely hope that the difficult technology to make it will not cause a too high price, to allow the broad general use of this – as far as I believe – valuable new drug [24].

Aspirin as a Household Remedy against Fever, Inflammation, and Pain Soon after the introduction of acetylsalicylic acid into medical use under the brand name "aspirin," the new drug became a most popular remedy against fever, inflammation, and pain. A local German newspaper (*Kölner Stadtanzeiger*) in the Leverkusen area published the following advice for treatment of flu on March 6, 1924:

. . . As soon as you feel yourself ill, you should go to bed and have a hot-water bottle at your feet. You should drink hot chamomilae tea or grog in order to sweat and should take 3 tablets of aspirin a day. If you follow these instructions you will recover within a few days, in most cases . . .

This citation is remarkable for several reasons: During the past 25 years of practical use, aspirin had become a drug whose name was well known not only to health professionals but also to the general public – and accepted without reservation by both. Certainly, the limited availability of antipyretic analgesics other than aspirin will have significantly contributed to this. The compound was recommended – and accepted – by both the lay press and the doctors – as a general "household remedy" for treatment of pain, fever, inflammation, and other kinds of "feeling bad," although very little if anything at all was known about the pharmacological mechanism of action behind these multiple activities (Section 1.1.3). Thus, in public opinion, a reliable medical effect for the user was considered much more important than an occasional dyspepsia or even the pharmacological reason for these effects. It took about half a century of intense practical use that the

first reports on clinically relevant (postoperative) bleeding were published (Section 1.1.4). However, at the time, an enhanced (gastro)intestinal blood loss and minor bleedings were already known as a typical side effect of salicylates. At daily doses of 1–3 g, about half of aspirin-treated patients were reported to have an estimated daily loss of 2–6 ml occult blood with the feces. Over 1 month, this amount was comparable to the blood loss during menstruation (50–100 ml) (Section 3.2.1) [26]. In addition, bloodletting was a frequently used therapeutic measure. Thus, bleeding was not considered a serious clinical problem by the vast majority of patients, particularly by those taking aspirin only once a day or occasionally in a few days to treat headache, flu, or other feverish discomfort.

Summary

The unlimited availability of entirely synthetic salicylic acid as a result of marked progress in organic chemistry in the late nineteenth century and the positive results with the compound in daily practice, including its medical use as an anti-inflammatory analgesic, eventually stimulated interest in chemical modifications. For medical use, the major aim was to improve the taste and (gastric) tolerance.

In this respect, the first successful synthesis of a pure, stable acetylated salicylic acid (aspirin) was performed by Felix Hoffmann in the group of Arthur Eichengrün at Bayer in Elberfeld in 1897. This research and further drug development were substantially supported by Carl Duisberg, the then Head of the research at Bayer, and resulted in a commercial preparation of an acetylsalicylic acid as tablet (Aspirin®) that was essentially free of unreacted salicylate and became the first industrial drug in tablet form worldwide.

After enthusiastic first reports about the clinical efficacy and tolerability – as opposed to salicylic acid – Aspirin® was launched in 1899 and rapidly became a well-known and well-accepted household remedy for treatment of fever, pain, inflammation, and other manifestations of "feeling bad." Side effects, except occasional gastric dyspepsia (nausea, vomiting), were rare at the time in the conventional short-term use, despite the rather high doses taken. Even the enhanced, about doubled, occult blood loss with the feces appeared not to be a serious problem except the very rare severe gastrointestinal bleedings.

References

1 Hangarter, W. (Hrsg.) (1974) Herkommen, Geschichte, Anwendung und weitere Entwicklung der Salizylsäure, in *Die Salizylsäure und ihre Abkömmlinge*, Schattauer, Stuttgart, pp. 3–11.

2 Gordonoff, T. (1965) Zur Geschichte der Antipyrese. *Wiener Med. Wochenschr.*, **115**, 45–46.

3 Gross, M. and Greenberg, L.A. (1948) *The Salicylates: A Critical Bibliographic Review*, Hillhouse Press, New Haven, CT.

4 Flechtner, H.-J. (1959) *Carl Duisberg – vom Chemiker zum Wirtschaftsführer*, Econ Verlag GmbH, Düsseldorf.

5 Stadlinger, H. (1947) Arthur Eichengrün 80 Jahre. *Die Pharmazie*, **2**, 382–384.

6 Eichengrün, A. (1949) 50 Jahre aspirin. *Die Pharmazie*, **4**, 582–584.

7 Dreser, H. (1899) Pharmakologisches über Aspirin (Acetylsalizylsäure). *Pflüg. Arch. Eur. J. Phy.*, **76**, 306–318.

8 McTavish, J.R. (1987) Aspirin in Germany: the pharmaceutical industry and the pharmaceutical profession. *Pharm. Hist.*, **29** (3), 103–115.

9 Busche, J. (2014) When Aspirin became American [Als Aspirin amerikanisch wurde]. *Magazin Heinrich-Heine Univ. Düsseldorf*, **3** (4), 20–21.

10 Mueller, R.L. and Scheidt, S. (1994) History of drugs for thrombotic disease: discovery, development, and directions for the future. *Circulation*, **89** (1), 432–449.

11 Jack, D.B. (1997) One hundred years of aspirin. *Lancet*, **350**, 437–439.

12 Starko, K.M. (2009) Salicylates and pandemic influenza mortality, 1918–1919 pharmacology, pathology, and historic evidence. *Clin. Infect. Dis.*, **49** (9), 1405–1410.

13 Sneader, W. (2000) The discovery of aspirin: a reappraisal. *BMJ*, **321** (7276), 1591–1594.

14 Wood, P.H.N. (1962) A milestone in the amelioration of pain: the man who invented Aspirin. *Medical News*, November 9.

15 Gerhardt, C.F. (1855) *Lehrbuch der Organischen Chemie. Deutsche Originalausgabe unter Mitwirkung von Prof. Dr. Rudolf Wagner*, Verlag Otto Wigand, Leipzig, p. 350.

16 Collier, H.O.J. (1963) Aspirin. *Sci. Am.*, **169**, 1–10.

17 Kraut, K. (1869) Ueber Salicylverbindungen. Mittheilungen aus dem Laboratorium der polytechnischen Schule zu Hannover. *Ann. Chemie Pharmac*, 150, 1–20.

18 Schlenk, O. (1947) Die salizylsäure, in *Arzneimittelforschungen 3*, Verlag Dr. Werner Saenger, Berlin.

19 Schlenk, O. (1947) Die Salizylsäure, In *Arzneimittelforschungen 3*, Verlag Dr. Werner Saenger, Berlin.

20 v. Gilm, H.A.d.P.-u.S. (1859) Acetylderivate der Phloretin- und Salicylsäure. *Liebigs Ann. Chem. Pharm.*, **112**, 180–182.

21 Schlenk, O. (1950) Bemerkung zur Arbeit "50 Jahre Aspirin" von Dr. Arthur Eichengrün, Pharmazie 4, 582 (1949). *Pharmazie*, **5**, 245.

22 Pace-Asciak, C. and Wolfe, L.S. (1971) A novel prostaglandin derivative formed from arachidonic acid by rat stomach homogenates. *Biochemistry*, **10** (20), 3657–3664.

23 Moncada, S. *et al.* (1976) An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263** (5579), 663–665.

24 Witthauer, K. (1899) Aspirin, ein neues Salicylpräparat. *Heilkunde*, **3**, 396–398.

25 Wolgemuth, J. (1899) Über Aspirin (Acetylsalicylsäure). *Ther Monatshefte*, **13**, 276–278.

26 Smith, M.J.H. and Smith, P.K. (eds.) (1966) *"The Salicylates": A Critical Bibliographic Review*, John Wiley & Sons, Inc., New York, p. 1–313.

1.1.3 Search for Pharmacological Modes of Action

In the monograph *The Salicylates*, Smith and Smith wrote: "The successful use of a drug in medicine is not precluded by a lack of knowledge about its mode of action . . . salicylates could be used as one of the better illustrations of [this] dictum . . ." [1]. This statement is further underlined by the fact that most drugs act at more than one site in a living organism. Thus, the molecular targets for salicylates might be similar or even the same at the cellular level but the consequences of an interaction with them may be quite different at the tissue and organ levels, respectively. In addition, aspirin is a unique compound, bearing two biologically active components in one and the same molecule: the reactive acetyl group and salicylic acid as the primary metabolite. As outlined in detail in Section 1.2, both components are completely different with respect to their targets, mode of action, and pharmacokinetic behavior.

In this chapter, the focus is on the three most historically important discoveries regarding the mode of action of aspirin: its effects on cellular energy metabolism as the first suggested – and confirmed – pharmacological mode of action of salicylates, the detection of inhibition of prostaglandin biosynthesis as the first pharmacodynamic action of aspirin with a relative specificity for aspirin over salicylate, and the interactions of aspirin with COX-2 at the transcriptional, translational, and posttranslational levels, including the generation of anti-inflammatory and inflammation-resolving compounds, such as the "aspirin-triggered" lipoxins.

1.1.3.1 Salicylates and Energy Metabolism of the Cell

Uncoupling of Oxidative Phosphorylation The first reports on basic pharmacology of aspirin and salicylates were published in the 1950s. One key finding was the detection of an interaction of salicylates with the energy metabolism of cells, that is, an increase in oxygen uptake subsequent to uncoupling of oxidative phosphorylation [2]. This effect, associated with marked depletion of cellular ATP levels and subsequent inhibition of ß-oxidation of fatty acids (Section 2.2.3), was seen at (low) millimolar concentrations of salicylates. These concentrations could be obtained by the intake of about 2–4 g aspirin per day, which was the conventional anti-inflammatory dose at the time. The uncoupling of energy due to energy-consuming processes clinically resulted in sweating and hyperventilation followed by metabolic acidosis in salicylate poisoning (Section 3.1.1). All of these actions are due to the salicylate metabolite of aspirin and, for the most part, can sufficiently be explained by the particular physicochemical properties of the compound [3–5] (Section 2.2.3).

ATP Depletion and Kinase Inhibition Exhaustion in ATP levels, that is, depletion of cellular energy stores, is a very fundamental event with considerable consequences for all energy-dependent functions of the cell. This includes cell proliferation, maintenance of the structure of the cytoskeleton, and, finally, cell survival. Biochemically, ATP depletion will inhibit or even completely prevent every kinase activation, that is, the phosphorylation of target enzymes and transcription factors, intimately involved in cell signaling and protein synthesis [6]. It is, however, difficult to understand how this rather nonselective mechanism can be selectively modified to restore the body homeostasis in pathologic conditions.

Thus, the explanation of the multiple pharmacological actions of aspirin by one ubiquitous mechanism – impaired energy metabolism by inhibition of oxidative phosphorylation – was an attractive and convincing concept at the time. Today it is considered to be primarily of toxicological interest because of the high concentrations required. There were clear pharmacological effects of aspirin on pain and inflammation at substantially lower doses, which did not disturb the cellular energy metabolism. In addition, it did not answer the question why aspirin – and other salicylates – preferably acted on inflamed or otherwise injured tissue but had no

clear-cut effects on healthy tissues. In addition, inhibition of oxidative phosphorylation by another well-known uncoupling agent, 2,4-dinitrophenol (DNP), had no anti-inflammatory action [7]. Thus, there was a clear need for a unique molecular target to explain the specific pharmacological effects of aspirin in inflammation and pain.

1.1.3.2 Aspirin and Prostaglandin Formation

Arachidonic Acid and Bradykinin Induce Pain and Smooth Muscle Contractions That Can Be Prevented with Aspirin A new and finally successful search for a more specific, that is, inflammation-related, mode of action of aspirin began with studies about pathomechanism, mediators, and symptoms of inflammation, specifically inflammatory pain as one of the most disturbing events. In 1959, it was shown by *R. Jaques* from the CIBA company in Basel (Switzerland) that pricking of diluted emulsions of low-dose arachidonic acid into the volar face of the human forearm caused pain, starting after a latency period of 15–20 s. This pain lasted for several minutes and was followed by a long-lasting (15–30 min) erythema without itching. *In vitro* experiments additionally showed that arachidonic acid at low concentrations (0.1 µg/ml) contracted the guinea pig ileum. Contractions again started after a latency period of 10–15 s and reached a peak after 45–90 s. They could be blocked by pretreatment with several agents, including analgesics such as aspirin (25 µg/ml), while salicylic acid, atropine, or mepyramine were inactive. This indicated that the contractions were not mediated by acetylcholine or histamine but, probably, by another chemical mediator.

From these and other data Jaques concluded that

> *Arachidonic acid . . . which is a constituent of body lipids or a substance with similar pharmacological characteristics . . . present in a preactive form might be set free by some enzyme system . . . and among other things cause pain* [8].

In 1969, another remarkable finding was published by Piper & Vane [9]. These authors showed that stimulation of isolated guinea-pig lung by bradykinin but not histamine caused release of "rabbit aorta contracting substance" (RCS) – later identified as a mix of prostaglandin endoperoxides and thromboxane - which was blocked by aspirin. Taken together, these findings suggested that aspirin prevented the generation rather than the action of hitherto unknown mediator(s) of pain and smooth muscle contraction. Similar findings were obtained in man after intra-arterial injection of bradykinin, a well-known pain producer, and it was additionally shown that its algesic effects could also be antagonized by aspirin [10]. However, the mode of algesic action of arachidonate and bradykinin as well as the analgesic action of aspirin remained unknown.

Inhibition of Prostaglandin Synthesis by Aspirin In 1971, the journal *Nature* published three papers of the group of *John R. Vane* (Figure 1.1.3-1), the then

Figure 1.1.3-1 Sir John R. Vane (from http://www.nobelprize.org/nobel_prizes/medicine/laureates/1982/vane-facts.html).

Professor of Pharmacology at the Royal College of Surgeons of England. In his pioneering article, Vane demonstrated for the first time a new mode of action of aspirin that was able to explain its anti-inflammatory and antipyretic actions by *one* single pharmacological mechanism: inhibition of (enhanced) biosynthesis of prostaglandins, a group of proinflammatory pain mediators [11].

*In his pioneering paper on aspirin and prostaglandins, the later Sir John Vane showed by as simple as elegant bioassay experiments that aspirin like indomethacin – and the somewhat less potent salicylate – inhibited prostaglandin formation in cell-free tissue homogenates of the guinea pig lung after addition of the natural precursor arachidonic acid – imitating endogenous arachidonic acid release after tissue injury (*Figure 1.1.3-2*). Vane suggested that the inhibition could be brought about by competition of these (acid) drugs with arachidonic acid for the active site of the prostaglandin-generating enzyme(s). He postulated that this mechanism accounts for the antipyretic and anti-inflammatory actions of salicylates but also their gastrointestinal symptoms by removal of a protective prostaglandin from the stomach mucosa. He, however, did not postulate that this was the explanation for all effects of anti-inflammatory drugs of the salicylate or indomethacin type. Specifically, he found no link between a peripheral analgesic action of these compounds and inhibition of prostaglandin synthesis.*

These findings of John Vane and the discovery of prostacyclin 5 years later were honored by the Nobel Prize for Medicine awarded to him in 1982.

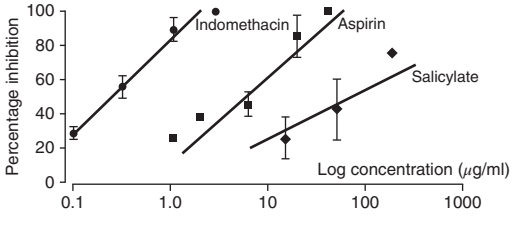


Figure 1.1.3-2 First description of inhibition of prostaglandin biosynthesis by aspirin and salicylate and the reference compound indomethacin. Note the dose dependency of the reaction with about 50% inhibition at <10 μg/ml (<60 μM) aspirin. (Modified after Ref. [11].)

A separate paper, published by *Bryan Smith and Al Willis* from the same laboratory on the following pages of the same issue of the journal, showed that a similar mechanism was also likely to work in human platelets. In this study, aspirin treatment of platelets *in vitro* or *ex vivo* nearly abolished thrombin-induced prostaglandin formation, although it did not affect the thrombin-induced "release reaction" of platelet-stored serotonin [12].

Prostaglandins and Other Eicosanoids Prostaglandins, thromboxane A_2, leukotrienes, and lipoxins are members of a group of natural lipid mediators that are all peroxidation products of arachidonic acid (5,8,11,14-all-*cis* eicosatetraenoic acid). Because of this, they all have a 20-carbon backbone and are summarized as "eicosanoids" (Greek: eikos = 20). Today, more than 150 eicosanoids are known and have been structurally identified. Arachidonic acid, the precursor fatty acid, is an essential constituent of the cell membrane phospholipids and is released from them by phospholipases. Prostaglandin synthesis starts with the availability of free arachidonic acid that occurs in close proximity to the metabolizing enzymes without requiring entry into the cytosol or the extracellular space.

The first oxidation step of arachidonic acid to generate prostaglandins is catalyzed by cyclooxygenases (COX). There are two isoforms of this enzyme – the constitutively expressed isoform COX-1 that is present in about every cell and tissue of the organism, and the inducible isoform COX-2, an "immediate early gene" that becomes upregulated in response to all kinds of stimuli, most notably humoral, immunological and inflammatory factors, and then accounts for the majority of prostaglandin formation. The primary oxidation products of both enzymes – the prostaglandin endoperoxides – are then converted to the terminal products of this pathway, that is, prostaglandins and thromboxane A_2, by different isomerases and synthases in a cell-specific manner. The local eicosanoid level is solely

controlled by biosynthesis. The active products are not stored but released upon stimulation, act on their cellular target via specific receptors, and are afterward rapidly degraded to inactive products by specific enzymes.

Prostaglandins exert their multiple actions via specific G-protein-coupled receptors, located at the cell surface. About 10 currently known prostaglandin receptors determine direction and intensity of the biological response that is highly organ and tissue specific. Prostaglandins act as local autocrine or paracrine mediators that dispatch signals between cells [13]. *Though prostaglandins can be formed in and act on probably all cells of the body, they are not essential for critical cell functions, such as energy metabolism or maintenance of the cell cytoskeleton, but rather act as modulators of cell functions.*

In a living organism, the cellular prostaglandin synthesis can be markedly increased in response to disturbed homoiostasis (injury) or humoral activation (sexual steroids, angiotensin) in order to locally adapt cell functions to changes in the environmental conditions. This increased prostaglandin synthesis "on demand" reflects a cell-specific response. Examples of physiological stimuli are hemostasis and pregnancy, whereas the increased prostaglandin production in inflammation,immune reactions, atherosclerosis, and tumorigenesis reflects the response to pathological stimuli.

Thus, any change in generation of prostaglandins or the related thromboxanes *per se* is neither good nor bad, but rather reflects functioning cell-based adaptation or defense mechanisms. Consequently, in functional terms, pharmacological interaction (inhibition) with prostaglandin formation may be either positive or negative. Functional disorders may arise when prostaglandins become critical factors for control of cell and organ function, respectively. However, in most cases, their removal is not associated with any measurable alterations at the organ level as long as other mediator systems can sufficiently compensate for it.

Inhibition of Cyclooxygenases Cyclooxygenases (COX) are the primary target of aspirin in the prostaglandin system. Aspirin blocks the biosynthesis of prostaglandins and thromboxane A_2 at the level of prostaglandin endoperoxide synthase (PGHS) or cyclooxygenase(s) (COX) by irreversible acetylation of a critical serine in the substrate channel of the enzyme (Section 2.2.1). This limits the access of substrate (arachidonic acid) to the catalytic active site within the substrate channel of the enzyme [14]. In 1975, the group of *Philip Majerus* detected the irreversible acetylation of the (platelet) cyclooxygenase (COX-1) by aspirin at $serine_{530}$ as the molecular site of action of aspirin, which is also the reason for inhibition of cyclooxygenase reactions in human platelets [15,16]. The group of *Garret FitzGerald* [17] confirmed this finding for the cloned enzyme from human platelets, while *William Smith* and coworkers described the molecular reaction kinetics [14]. These and other major contributions to the issue were acknowledged with the Aspirin Senior Award to William Smith in 1997. The molecular interaction of aspirin with COX-1 was further elucidated after the crystal structure of the enzyme became elucidated by *Michael Garavito* and coworkers. The contribution of *Patrick Loll* of his group to this work was acknowledged with the Young Researchers' Aspirin Award [18].

The detection of inhibition of prostaglandin synthesis was the first plausible explanation for the multiple pharmacological actions of aspirin in inflammation, pain, fever, and hemostasis via one ubiquitous class of endogenous mediators, prostaglandins and thromboxanes. With increasing knowledge of the complex nature of these reactions, specifically after detection of the multiple interactions of prostaglandins with other mediator systems, details of these findings are now interpreted in a different way. This is particularly valid for the numerous and complex effects of aspirin and salicylate on gene transcription (see below) [19].

1.1.3.3 Aspirin and COX-2

Acetylation of COX-2 by Aspirin Both COX-1 and COX-2 isoforms are molecular targets for aspirin and both become acetylated by aspirin at the same serine site inside the fatty acid substrate channel ($serine_{530}$ in COX-1 but $serine_{512}$ in COX-2

because of the missing 18 amino acids) (for details, see Section 2.2.1). However, the consequences of this serine acetylation are different. The inhibition of the constitutive COX-1 in platelets and other cells is a posttranslational and irreversible acetylation response, which cannot be obtained with salicylate. In contrast, the consequences of acetylation of COX-2 by aspirin are more complex. They mainly result in conversion of the enzyme activity toward a 15-lipoxygenase. Moreover, the inhibition of prostaglandin production by COX-2 *in vivo* is probably never really complete because of the inducibility of enzyme protein by about any kind of stimuli and the short turnover rate of the enzyme protein. Very recently, the crystal structure of the acetylated human COX-2 has been elucidated by *Michael J. Lucido* and coworkers from *Michael G. Malkowskís* group. Major findings were that the acetylated $serine_{530}$ in COX-2 completely blocks access of substrate to the hydrophobic side pocket and that salicylate binding is reflective of an enzyme–inhibitor complex prior to acetylation [20]. COX-2 has also a much broader substrate specificity, including arachidonylethanolamide (anandamide) from which prostamides can be formed [1]. Interestingly, acetylated COX-2 also generates entirely new classes of lipid mediators, that is, 15-(*R*)-HETE, a precursor of anti-inflammatory lipoxins. For more details, see Section 2.2.1 (Figure 2.2.1-1).

Lipoxins The main effect of aspirin on COX-2 is rather a modulation than (complete) an inhibition of enzyme activity, resulting in generation of 15-(*R*)-hydroxyeicosatetraenoic acid (15-(*R*)-HETE). This fatty acid is substrate for subsequent generation of "aspirin-triggered lipoxin" (ATL) by interaction with the 5-lipoxygenase from white cells (Sections 2.2.1 and 2.3.2). Lipoxins and the related resolvins represent new classes of anti-inflammatory mediators that are formed by interaction of aspirin with COX-2 but not by any other nonsteroidal anti-inflammatory agents.

Aspirin-triggered lipoxin (15-*epi*-LTA_4) was first described by *Charles Serhan* and his group. The contributions of *Joan Claria* to this work [22] were acknowledged with the Aspirin Junior Award in 1996. More recent work by *Derek Gilroy* and his group demonstrated that aspirin even at low antiplatelet doses of 75 mg/day stimulates the generation of anti-inflammatory 15-*epi*-LTA_4 and inhibits white cell accumulation in inflammatory skin exudates of men at only minor reduction of PGE_2 levels [23] (Section 2.3.2). This finding was the first to show an anti-inflammatory effect of aspirin via modulation rather than inhibition of COX-2, which was possibly also related to the antiplatelet effect of aspirin. This fundamental finding was acknowledged with the Young Researchers' Aspirin Award in 2005.

Salicylates and Gene Transcription *Kenneth K. Wu* (Figure 1.1.3-3) and his group were the first to show that both aspirin and salicylate can interact with the binding of transcription factors to the promoter region of the COX-2 gene. In tissue injury, transcription factors regulate gene expression levels after stimulation by inflammatory

Figure 1.1.3-3 Kenneth K. Wu.

mediators in many cell types, including white cells and endothelial cells [24,25]. Later work of this group eventually identified salicylate-induced phosphorylation of CCAAT/enhancer-binding protein-β (C/EBP-β or NF/IL-6) as the critical factor [19,26]. Thus, anti-inflammatory actions of aspirin or salicylate may also involve inhibition of transcription of the COX-2 gene, followed by reduced production of enzyme protein. This occurs in addition to translational and post-translational modifications, that is, inhibition of prostaglandin synthesis by inhibition of enzyme activity. Interestingly, as also shown by this group, control of gene transcription by aspirin and salicylate is not restricted to COX-2, rather it has also been shown for other immediate early genes that are regulated by the same transcription factors, such as the inducible NO synthase (iNOS), generating large amounts of NO, another most important inflammatory mediator (Section 2.2.2).

In this context, it is interesting to note the role of salicylates in plants – their natural sources. In many plants, salicylates represent a defence system of vital importance that becomes activated by gene transcription in response to about every kind of noxious stimuli and exhibits a number of similarities to the prostaglandin pathway in animals and men (Section 2.2.2).

Summary

In the 1950s, uncoupling of oxidative phosphorylation was described as the first cellular mode of action of salicylates. These metabolic effects required high local concentrations of the compounds, frequently in the millimolar range, and lacked specificity for inflamed or otherwise injured tissue. They, however, did explain the metabolic effects of high-dose salicylate (hyperventilation, sweating, and metabolic acidosis) as well as intoxication.

In 1971, Sir John Vane suggested for the first time inhibition of prostaglandin synthesis by aspirin as a uniform mode of its antipyretic and anti-inflammatory actions. This finding explained the clinical results in treatment of fever and inflammatory pain. In molecular terms, this was later explained by specific and irreversible acetylation of a serine in the substrate channel of platelet cyclooxygenase (COX), upstream to the active site of the enzyme.

After detection of two genetically defined isoforms of cyclooxygenases – COX-1 and COX-2, it became apparent that the molecular mode of action of aspirin is the same on both enzymes – irreversible acetylation of a target serine – but the consequences are different. In COX-1, this results in largely complete inhibition of prostaglandin and thromboxane formation. This is most effective in platelets that are unable to replace the acetylated COX-1 by fresh enzyme protein because of their very low protein-synthesizing capacity. In COX-2, an enzyme with a much broader substrate specificity, acetylation by aspirin also causes inhibition of prostaglandin production but mainly modulation of enzyme activity toward a 15-lipoxygenase. This eventually results in the generation of 15-(*R*)-HETE, a precursor for aspirin-triggered lipoxin and other anti-inflammatory mediators. Additionally, both aspirin and salicylate inhibit the cytokine-induced expression of COX-2 and other immediate early genes, such as iNOS, at the transcriptional level.

References

1 Smith, M.J.H. and Smith, P.K. (eds.) (1966) *"The Salicylates": A Critical Bibliographic Review*, John Wiley & Sons, Inc., New York, pp. 1–313.

2 Brody, T.M. (1956) Action of sodium salicylate and related compounds on tissue metabolism *in vitro*. *J. Pharmacol. Exp. Ther.*, **117** (1), 39–51.

3 Furst, D.E., Gupta, N., and Paulus, H.E. (1977) Salicylate metabolism in twins: evidence suggesting a genetic influence and induction of salicylurate formation. *J. Clin. Invest.*, **60** (1), 32–42.

4 Whitehouse, M.W. (1964) Biochemical properties of anti-inflammatory drugs–III. Uncoupling of oxidative phosphorylation in a connective tissue (cartilage) and liver mitochondria by salicylate analogues: relationship of structure to activity. *Biochem. Pharmacol.*, **13**, 319–336.

5 Whitehouse, M.W. (1964) Uncoupling of oxidative phosphorylation by some arylacetic acids (anti-inflammatory or hypercholesterolemic drugs). *Nature*, **201**, 629–630.

6 Frantz, B. and O'Neill, E.A. (1995) The effect of sodium salicylate and aspirin on NF-kappa B. *Science*, **270** (5244), 2017–2019.

7 Marks, V. and Smith, M.J. (1960) Anti-inflammatory activity of salicylate. *Nature*, **187**, 610.

8 Jaques, R. (1959) Arachidonic acid, an unsaturated fatty acid which produces slow contractions of smooth muscle and causes pain: pharmacological and biochemical characterisation of its mode of action. *Helv. Physiol. Pharmacol. Acta.*, **17**, 255–267.

9 Piper, P.J. and Vane, J.R. (1969) Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature*, **223** (5201), 29–35.

10 Coffman, J.D. (1966) The effect of aspirin on pain and hand blood flow responses to intra-arterial injection of bradykinin in man. *Clin. Pharmacol. Ther.*, **7** (1), 26–37.

11 Vane, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.*, **231** (25), 232–235.

12 Smith, J.B. and Willis, A.L. (1971) Aspirin selectively inhibits prostaglandin production in human platelets. *Nat. New Biol.*, **231** (25), 235–237.

13 Hirata, T. and Narumiya, S. Prostanoid receptors. *Chem. Rev.*, **111** (10), 6209–6230.

14 DeWitt, D.L. *et al.* (1990) The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J. Biol. Chem.*, **265** (9), 5192–5198.

15 Roth, G.J. and Majerus, P.W. (1975) The mechanism of the effect of aspirin on human platelets: I. Acetylation of a particulate fraction protein. *J. Clin. Invest.*, **56** (3), 624–632.

16 Roth, G.J., Stanford, N., and Majerus, P.W. (1975) Acetylation of prostaglandin synthase by aspirin. *Proc. Natl. Acad. Sci. USA*, **72** (8), 3073–3076.

17 Funk, C.D. *et al.* (1991) Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *FASEB J.*, **5** (9), 2304–2312.

18 Loll, P.J., Picot, D., and Garavito, R.M. (1995) The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase. *Nat. Struct. Biol.*, **2** (8), 637–643.

19 Saunders, M.A. *et al.* (2001) Selective suppression of CCAAT/enhancer-binding protein beta binding and cyclooxygenase-2 promoter activity by sodium salicylate in quiescent human fibroblasts. *J. Biol. Chem.*, **276** (22), 18897–18904.

20 Lucido, M.J. *et al.* (2016) The crystal structure of aspirin acetylated human cyclooxygenase-2: insight into the formation of products with reversed stereochemistry. *Biochemistry*. doi: 10.1021/acs.biochem.5b01378

21 Yu, M., Ives, D., and Ramesha, C.S. (1997) Synthesis of prostaglandin E2 ethanolamide from anandamide by cyclooxygenase-2. *J. Biol. Chem.*, **272** (34), 21181–21186.

22 Claria, J. and Serhan, C.N. (1995) Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell–leukocyte interactions. *Proc. Natl. Acad. Sci. USA*, **92** (21), 9475–9479.

23 Morris, T. *et al.* (2009) Effects of low-dose aspirin on acute inflammatory responses in humans. *J. Immunol.*, **183** (3), 2089–2096.

24 Wu, K.K. *et al.* (1991) Aspirin inhibits interleukin 1-induced prostaglandin H synthase expression in cultured endothelial cells. *Proc. Natl. Acad. Sci. USA*, **88** (6), 2384–2387.

25 Xu, X.M. *et al.* (1999) Suppression of inducible cyclooxygenase 2 gene transcription by aspirin and sodium salicylate. *Proc. Natl. Acad. Sci. USA*, **96** (9), 5292–5297.

26 Cieslik, K.A. *et al.* (2005) Inhibition of p90 ribosomal S6 kinase-mediated CCAAT/enhancer-binding protein beta activation and cyclooxygenase-2 expression by salicylate. *J. Biol. Chem.*, **280** (18), 18411–18417.

1.1.4 Clinical Applications: A Piece of History

1.1.4.1 Anti-Inflammatory/Analgesic Actions

The disclosure of a causal relationship between inhibition of prostaglandin synthesis and the antipyretic and anti-inflammatory actions of aspirin provided an attractive explanation for its therapeutic efficacy in the treatment of inflammatory and feverish diseases. It also stimulated mechanism-based research for new anti-inflammatory compounds. These compounds were designed to block prostaglandin biosynthesis but should be more potent than aspirin in order to allow lower dosing. Indomethacin was the first of these so-called "aspirin-like drugs" [1] and was already used as a reference compound in the pioneering experiments of Sir John Vane (Figure 1.1.3-2). Many others followed. In 2009, there were more than 20 chemicals alone on the German market, which were developed as reversible-type inhibitors of prostaglandin biosynthesis and were approved for clinical use as antipyretic/anti-inflammatory analgesics. This included more than 40 (!) brands containing ibuprofen, 19 containing diclofenac, and 4 containing indomethacin, most of them available in different galenic formulations and doses. On the other hand, there were 10 brands containing acetylsalicylic acid as the only active ingredient. Only one single agent has been developed but not yet introduced clinically: 2-(acetoxy-phenyl)hept-2-ynyl sulfide (APHS), which mimicked the irreversible mode of COX–serine–acetylation of aspirin and exhibited significant COX-2 selectivity [2,3]. Thus, the invention of aspirin has markedly stimulated basic research for new anti-inflammatory analgesics – and still does – as evident from the detection of aspirin-triggered lipoxins and resolvins. However, aspirin has lost its outstanding position as a *medicine* for treatment of chronic inflammatory diseases, such as rheumatoid arthritis – the indication it was originally introduced for into the clinics (Section 4.2.2). In addition to its use in cardiovascular prevention (see below) its, use as a putative *chemopreventive* is steadily increasing. According to a recent survey, regular intake of aspirin has increased by 60% from 2005 to 2010, that is, to 43.6 million people alone in the United States. In 2005, 25% of these individuals took aspirin for prevention of colorectal cancer; in 2010, these were 42%. This is a figure similar to the percentage taking aspirin for cardiovascular prevention [4].

Through the first 50 years of its practical use, aspirin became a well-accepted and increasingly used anti-inflammatory/antipyretic OTC analgesic worldwide (Figure 1.1.4-1). Today, the compound in its numerous commercial and galenic formulations still belongs to the most frequently used antipyretic analgesics for self-medication of headache, flu, and other acute inflammatory/painful conditions. According to a German registry (MONICA registry), salicylates still keep a key position (70–80%) in OTC drugs for these indications [5]. Actually, aspirin has to compete in these indications with ibuprofen and the non-anti-inflammatory acetaminophen, but it still does very well [6]. Regarding the pharmacological mode of action, there are distinct advantages and disadvantages with either of these compounds in OTC use, which are discussed in more detail in the clinical Sections 4.1–4.3.

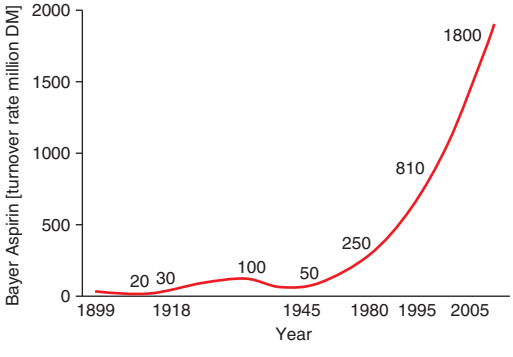


Figure 1.1.4-1 Turnover rates for Bayer Aspirin from 1899 to 2005 (projected) in millions of DM (Deutsche Mark). 1 DM is equivalent to €0.51 or about $0.7. (After Ref. [5]).

1.1.4.2 Antiplatelet/Antithrombotic Actions and the Bleeding Tendency

Aspirin and Bleeding As mentioned earlier (Section 1.1.2), bleedings were not considered a serious clinical problem by the vast majority of individuals, who took aspirin only once or for a few days to treat headache and symptoms of flu or other feverish discomfort. The compound was in clinical use for about half a century, when first reports about bleedings as a possible clinical problem were published. In 1945, *Singer*, an ETN-physician, reported late bleedings after tonsillectomies. He attributed this to prescription of aspirin for analgesic purposes. No bleedings of this type were seen when aspirin was withdrawn or replaced by the non-anti-inflammatory analgesic metamizol (dipyrone) [7]. Prolonged bleeding was also seen in cardiac patients who were aspirin treated [8]. A relationship between aspirin intake and bleeding was also suggested for other minor surgeries, such as tooth extraction [9].

The first mechanistic concepts were developed by relating the bleeding tendency after aspirin to thrombin and the coagulation system [10]. *Armand J. Quick and L. Clesceri* [11] were among the first to suggest a connection between the two. They assumed that the bleeding tendency after high-dose (6 g) aspirin might be due to diminution of a stable procoagulatory factor in plasma, probably prothrombin. In 1966, Quick also demonstrated that a prolonged bleeding time *ex vivo* at lower doses was specific for aspirin and was not seen with salicylate [11,12] (Figure 3.1.2-1). Interestingly, aspirin did not prolong clotting time *in vitro* [13], suggesting the existence of additional aspirin-sensitive factors for prolongation of bleeding time *in vivo* (Section 3.1.2)

Aspirin and Antiplatelet/Antithrombotic Effects In 1967/1968, several groups showed independent of each other that within the clotting system, platelets were the target of interest for aspirin and that aspirin inhibited various aspects of platelet function. *H. Klaus Breddin* showed that aspirin treatment of patients with peripheral arterial occlusive disease resulted in inhibition of platelet aggregation and a prolongation of bleeding time by nearly 2 min after intake of 500 mg [14]. *Harvey J. Weiss* found that only aspirin but not salicylate inhibited ADP release and "secondary platelet aggregation" and that this reaction was irreversible during the platelet life span [13]. Later this group also showed that inhibition of platelet function resulted in disturbed platelet thrombus formation and thrombus adhesion to the subendothelium [15]. The group of *James F. Mustard* [16] further demonstrated that inhibition of platelet aggregation by aspirin was dependent on the kind of platelet stimulation. Thus, even a high dose of 1 mg/ml (!) aspirin did not block high-dose thrombin-induced platelet aggregation, but did so when low-dose thrombin was used. This probably explains the missing inhibition of platelet function by aspirin, later reported by Smith and Willis [17]. Alternatively, this might also suggest the requirement of additional platelet-stimulating factors, released or generated from platelets by thrombin, such as ADP – or thromboxane A_2 that was not known at the time – for a full response. In addition, aspirin did not inhibit ADP-induced *primary* platelet aggregation, that is, contraction of the platelet cytoskeleton, but only the ADP-induced secretion response. This was confirmed by others after oral administration of aspirin to man. In his study, *John R. O'Brien* found a "permanent" inhibition of platelet function by aspirin at a "subclinical" single dose of 150 mg and strongly recommended a clinical trial of the compound in patients at elevated thrombotic risk to determine its therapeutic potential. He also noted that this aspirin-induced platelet "abnormality" could be corrected by addition of 10% untreated platelets [18].

Detection of a Mode of Action and the First Clinical Trials *Philip W. Majerus* and his group were the first to show that aspirin irreversibly binds to a particular protein fraction (COX) and acetylates

a specific serine (serine$_{530}$) inside the COX-channel [19,20]. He also initiated the first double-blind, placebo-controlled clinical trial to study antithrombotic effects of an antiplatelet dose (160 mg/day) of aspirin in hemodialysis patients at risk for thrombotic shunt occlusion [21] (Section 4.1.1). Some details of the study design and results were written down 25 years later by Dr. Majerus and are still interesting to read:

> *. . . This study was done in 1978 when clinical trials were much easier to carry out than it is today. Late one afternoon, I looked into the St. Louis phone directory for aspirin and found a company in town, Rexall, that made aspirin tablets. I called after hours, and a man answered the phone. I explained what I wanted: 100 bottles of 100 tablets containing 160 mg aspirin and the same number of bottles of a matched placebo. The man said he could make them without any problem and he delivered them to my lab next morning at no charge. We continued the study for 6 months by which time 18 of 25 patients in the placebo group had a thrombosis compared to 6 of 19 of those given aspirin for a relative reduction of 3-fold, a highly significant result . . .* [22].

This first clinical trial was followed by others, done independently by several groups at about the same time. They provided the rationale for inhibition of platelet function by aspirin as a treatment option to prevent atherothrombotic vessel occlusion, specifically myocardial infarction and ischemic stroke (Sections 4.1.1 and 4.1.2). However, other diseases with a pathology in blood clotting also came into focus. This included pregnancy-induced hypertension (preeclampsia) (Section 4.1.5). The first description of positive effects of aspirin came from *Alex J. Crandon and David M. Isherwood* [23] and was confirmed in a randomized trial by *Michel Beaufils et al.* 6 years later [24]. The early use of aspirin in prevention of preeclampsia (pregnancy induced hypertension) in high-risk pregnancies is now a worldwide considered option by the WHO [25], also because there are no real pharmacological alternatives and aspirin belongs to the very few agents with a very low, if any, drug-related fetal toxicity (Section 4.1.5).

1.1.4.3 Aspirin and the History of Prevention of Myocardial Infarction and Stroke

First Observations In 1948/1949, *Paul C. Gibson* (London, UK) published the first reports on successful use of aspirin for prevention of angina pain and coronary thrombosis in patients, suffering from various thrombotic diseases. In 1949 he published data based on a questionnaire, sent by him to 20 doctors who treated cardiac patients. The majority of them considered aspirin as being of "undoubted value" in relieving and preventing angina pain while none thought it was useless. Gibson explained these beneficial effects by a combination of coumarin-like and analgesic effects of the compound. He recommended doses of 1300 mg (20 grains) every 4 h for 10 days, followed by 650 mg (10 grains) aspirin "as long as desirable" for patients with myocardial infarction [26].

The Craven Studies The first systematic investigations on the significance of antithrombotic effects of aspirin for prevention of myocardial infarction and stroke were published by *Lawrence L. Craven* (Figure 1.1.4-2), a suburban General Practitioner from Glendale (California) [27]. His studies were initiated by the fact that coumarin-type anticoagulants (dicumarol) had already been very successfully used at the time for prevention of coronary thrombosis, that is, myocardial infarctions [28]. Though the clinical efficacy of dicumarol was impressive, there were several problems with its practical handling. The percentage of time when patients were at the desired therapeutic level was suboptimal and either too short (low) (ineffective) or too long (high) (bleeding). This was assumed to be due to insufficient treatment control abilities by physicians and laboratories and/or the patient's compliance rather than due to a failure of the drug to act [29]. Salicylate had been previously identified as an intermediate in the metabolism of coumarins in men [30]. This apparently explained the anticoagulant action of aspirin and the absent

Figure 1.1.4-2 Photograph of Dr. Lawrence L. Craven in 1914, at the age of 31, when he graduated from the University of Minnesota College of Medicine and Surgery. (Courtesy of University of Minnesota.)

effects of coumarins (dicumarol) on bleeding time *in vitro*. Moreover, studies of the pharmacological activity of various analogues of dicumarol had revealed that only those compounds showed anticoagulant action *in vivo* that theoretically could yield salicylic acid or a derivative thereof on metabolic processing in vivo [31]. On this background, Craven, similar to Gibson, assumed that aspirin (salicylate) could be considered a less potent – but better tolerated – substitute for dicumarol and in 1948 started his systemic studies on aspirin as a preventive of myocardial infarction. We now know that there is no pharmacokinetic connection between coumarins and aspirin at antiplatelet doses (Section 2.3.1.4). Thus, the hypothesis of using aspirin as a warfarin replacement (warfarin-light) for prevention of myocardial infarction was wrong – however, with marked clinical consequences. Indeed, aspirin turned out to be very effective in prevention of myocardial infarctions in these studies.

Craven summarized the findings of his first study as follows:

> *. . . during the past two years, I have advised all of my male patients between the ages of 40 and 65 to take from 10–30 grains [650–1950 mg] of acetylsalicylic acid daily as a possible preventive of coronary thrombosis. More than 400 have done so, and of these none has suffered a coronary thrombosis. From past experience, I should have expected at least a few thrombotic episodes among this group. There would appear to be enough evidence of the antithrombotic action of acetylsalicylic acid to warrant further study under more carefully controlled conditions . . .* [32].

Craven continued these studies and reportedly treated 1465 patients until 1953 – still without having seen any unstable angina attack or myocardial infarction. Meanwhile, he had reduced the daily dose to 5 grains = 325 mg or one tablet per day – a dose still frequently used in cardiovascular prevention. Craven was aware of the uncontrolled nature of his studies including missing untreated control patients and stated:

> *. . . in such a large number of subjects of this type most likely to experience coronary episodes it is – to say the least – remarkable that all remained healthy and active. Such a finding is contrary to statistical expectations as well as to the consistent experience of 36 years in general practice . . . .*

He concluded:

> *. . . will experimental and clinical research in its slow but steady progress eventually test the observations here presented? Only the future can tell whether they are finally to be substantiated or refuted . . .* [33]

In the following years, Craven increased the number of patients to about 8000 – still without having seen any myocardial infarction – and recommended the drug also for prevention of stroke [33,34]. The recommended daily dose was now 5–10 grains = 325–650 mg = 1–2 tablets per

day. Unfortunately, he died himself in 1957, a year after publication of his last study, at the age of 74 years from a heart attack. It is not known from his records of whether or not he has followed his own advices to take 1 aspirin tablet per day. One reason for *not* taking it could be that according to his opinion, aspirin had a bad benefit–risk profile at the age of over 70 years – he had recommended the drug only for middle-aged (40–65 years) men [35].

These studies of Craven were the first to suggest beneficial effects of aspirin for prevention of myocardial infarction and stroke. However, they were also a stroke of luck for several reasons: Thus, Craven treated exclusively males at an age of increased risk for myocardial infarction who, according to current knowledge, benefit most from aspirin prophylaxis. He used a dose of aspirin – 325 mg = 1 tablet per day – which was very low in comparison to anti-inflammatory doses used at the time for treatment of chronic inflammatory diseases. Thus, not too many side effects had to be expected, which was also good for the compliance of his patients. Finally, he had no problems with statistics because there were no infarctions in any of the patients' groups.

Consequences of the Craven Studies Unfortunately, these exciting findings, including a once-daily 325 mg aspirin tablet that had proven to be effective, did not find due attention during the following 20 years. This was possibly influenced by the low impact factor of the journals where these studies were published and the fact that Craven himself died of heart attack. Until the 1970s, the pathology of myocardial infarction was also unclear, that is, whether a coronary thrombosis was a cause or consequence of an infarction. *Peter C. Elwood*, *Archibald L. Cochrane* and colleagues published in 1974 the first randomized, placebo-controlled trial on aspirin (300 mg/day) in 1239 men with recent myocardial infarction. They found a reduced mortality of 25% after 12 months, which, however, was not significant. Consequently, the authors considered their results as being inconclusive [36]. Until 1988, more than 15 000 patients were studied in seven placebo-controlled trials for secondary prevention of myocardial infarction at the cost of many millions of dollars. None of these studies was significant on its own. Possible explanations from today's viewpoint are a poor study design, highly variable aspirin doses (300–1500 mg/day), the apparently largely if not totally absent control of patients' compliance over the months and years of study duration, and a highly variable time point at which aspirin treatment was started, in one study (AMIS) up to 5 years (!) after the acute event [37].

These negative results at first finished the discussion on aspirin use for prevention of myocardial infarction. In addition, infrequent though severe side effects, such as GI or cerebral bleeding, and a suggested although never really established relationship with Reye's syndrome (Section 3.3.3) have tainted its reputation and resulted in removal of aspirin from the list of essential drugs by the WHO in 1988.

Aspirin Revival in Thrombosis Prevention Ironically, in the same year 1988, when the WHO took action to remove aspirin from the list of essential drugs, two large prospective, randomized, and placebo-controlled trials were published that convincingly demonstrated a cardioprotective action of regular low-dose aspirin: one tablet (325 mg) every second day or half a tablet, that is, 162 mg, daily – the US-American Physicians' Health study (US-PHS), showing a 44% reduction in the occurrence of a first myocardial infarction in a healthy population [38], and the ISIS-2 trial, showing a 50% reduction in reinfarctions and a 23% reduction in mortality over 5 weeks in patients with acute myocardial infarction [39] (Section 4.1.1). *Charles H. Hennekens*, then at Harvard Medical School (Boston), was a leading figure in both of these keynote trials. As a consequence of ISIS-2 and supported by the publication of the first of a series of meta-analyses by the Antiplatelet

Trialists' Collaboration on secondary prevention of vascular diseases [40], aspirin became the official guideline recommendation for patients with acute coronary syndromes. It still keeps this position, now in combined use with other antiplatelet agents, such as ADP receptor antagonists, as antiplatelet treatment of choice in this clinical condition.

Summary

The discovery of a general mode of action of aspirin – inhibition of prostaglandin biosynthesis – had considerable consequences for its clinical use and resulted in the systematic development of the new class of nonsteroidal anti-inflammatory analgesics (NSAIDs) with the common feature of inhibition of prostaglandin biosynthesis. These have largely replaced aspirin in symptomatic long-term treatment of chronic inflammatory diseases like rheumatism, but not as an OTC antipyretic analgesic. The compound kept its unique position until today – although now in competition with other non-opioid analgesics, such as ibuprofen and the non-anti-inflammatory acetaminophen (paracetamol).

In the mid-1960s, the first papers were published showing prolongation of bleeding time and inhibition of platelet function by aspirin. These effects were irreversible, not seen with other salicylates, and (later) explained by inhibition of prostaglandin (thromboxane) biosynthesis. The extent of inhibition was dependent on the platelet stimulus, involving apparently all aspects of platelet function, including adhesion to the (sub)endothelium, and could be prevented by addition of a small amount (10%) of untreated, aspirin "naïve" platelets. In 1979, the first placebo-controlled clinical trial demonstrated a significant inhibition of shunt thrombosis by 160 mg/day aspirin in patients on chronic hemodialysis.

Lawrence L. Craven was the first physician to report positive results with aspirin in prevention of myocardial infarction and stroke. He gave the drug to several thousands of middle-aged male persons, finally at a dose of 325 mg, that is, "1 tablet per day" and, reportedly, did not see any myocardial infarction over years. A number of follow-up studies could not confirm these results, most likely due to insufficient study protocols, until 1988 when the Physicians' Health study in primary and the ISIS-2 study in secondary prevention were published. Both studies showed marked reductions of myocardial infarction rates and improved infarct survival, respectively, with only 1 aspirin tablet (325 mg) every second day or half-a tablet every day.

References

1 Shen, T.Y. (1981) Burger Award address. Toward more selective antiarthritic therapy. *J. Med. Chem.*, **24**, 1–5.

2 Kalgutkar, A.S. *et al.* (1998) Covalent modification of cyclooxygenase-2 (COX-2) by 2-acetoxyphenyl alkyl sulfides, a new class of selective COX-2 inactivators. *J. Med. Chem.*, **41** (24), 4800–4818.

3 Kalgutkar, A.S. *et al.* (1998) Aspirin-like molecules that covalently inactivate cyclooxygenase-2. *Science*, **280** (5367), 1268–1270.

4 Zhou, Y., Boudreau, D.M., and Freedman, A.N. (2013) Trends in the use of aspirin and nonsteroidal anti-inflammatory drugs in the general U.S. population. *Pharmacoepidemiol. Drug Saf.*, **23** (1), 43–50.

5 Schenkirsch, G. *et al.* (2001) [Ten-year trend of analgesic drug use (1984 to 1995): results of three MONICA-based surveys in Augsburg)]. *Dtsch. Med. Wochenschr.*, **126** (22), 643–648.

6 Baron, J.A. *et al.* (2013) Gastrointestinal adverse effects of short-term aspirin use: a meta-analysis of published randomized controlled trials. *Drugs R D*, **13** (1), 9–16.

7 Singer, R. (1945) Acetylsalicylic acid, a probable cause for secondary post-tonsillectomy hemorrhage: a

preliminary report. *Arch. Otolaryngol, (Chicago)*, **42**, 19–20.
8 Beaumont, J.L. and Willie, A. (1955) Influence sur l'hémostase, de l'hypertension artérielle, des antivitamines K, de l'héparine et de l'acide acétyl salicylique. *Sang*, **26**, 880.
9 Smith, J.M. and Mackinnon, J. (1951) Aetiology of aspirin bleeding. *Lancet*, **2** (6683), 569–570.
10 Wising, P. (1952) Haematuria, hypoprothrombinemia and salicylate medication. *Acta. Med. Scand.*, **141**, 256.
11 Quick, A.J. and Clesceri, L. (1960) Influence of acetylsalicylic acid and salicylaide on the coagulation of blood. *J. Pharmacol. Exp. Ther.*, **128**, 95–98.
12 Quick, A.J. (1966) Salicylates and bleeding: the aspirin tolerance test. *Am. J. Med. Sci.*, **252** (3), 265–269.
13 Weiss, H.J., Aledort, L.M., and Kochwa, S. (1968) The effect of salicylates on the hemostatic properties of platelets in man. *J. Clin. Invest.*, **47** (9), 2169–2180.
14 Breddin, H.K. (1968) Discussion remarks: actions of drugs on platelet aggregation [Wirkung von Pharmaka auf die Plättchenaggregation]. *Verhandlungen der Deutschen Arbeitsgemeinschaft für Blutgerinnungsforschung anläßlich der 11. Tagung in Wien, April 14–15, 1967. Schattauer, Stuttgart*, pp. 90–95.
15 Weiss, H.J., Tschopp, T.B., and Baumgartner, H.R. (1975) Impaired interaction (adhesion–aggregation) of platelets with the subendothelium in storage-pool disease and after aspirin ingestion: a comparison with von Willebrand's disease. *N. Engl. J. Med.*, **293** (13), 619–623.
16 Evans, G. *et al.* (1968) The effect of acetylsalicylic acid on platelet function. *J. Exp. Med.*, **128** (5), 877–894.
17 Smith, J.B. and Willis, A.L. (1971) Aspirin selectively inhibits prostaglandin production in human platelets. *Nat. New Biol.*, **231** (25), 235–237.
18 O'Brien, J.R. (1968) Effects of salicylates on human platelets. *Lancet*, **1**, 779–783.
19 Roth, G.J. and Majerus, P.W. (1975) The mechanism of the effect of aspirin on human platelets: I. Acetylation of a particulate fraction protein. *J. Clin. Invest.*, **56** (3), 624–632.
20 Roth, G.J. and Siok, C.J. (1978) Acetylation of the NH_2-terminal serine of prostaglandin synthetase by aspirin. *J. Biol. Chem.*, **253** (11), 3782–3784.
21 Harter, H.R. *et al.* (1979) Prevention of thrombosis in patients on hemodialysis by low-dose aspirin. *N. Engl. J. Med.*, **301** (11), 577–579.
22 Majerus, P.W. (2014) An aspirin a day. *Adv. Biol. Regul.*, **54**, 231–241.
23 Crandon, A.J. and Isherwood, D.M. (1979) Effect of aspirin on incidence of pre-eclampsia. *Lancet*, **1** (8130), 1356.
24 Beaufils, M. *et al.* (1985) Prevention of preeclampsia by early antiplatelet therapy. *Lancet*, **1**, 840–842.
25 WHO (2011) *WHO Recommendations for Prevention and Treatment of Pre-Eclampsia and Eclampsia*, WHO Press.
26 Gibson, P.C. (1949) Aspirin in the treatment of vascular diseases. *Lancet*, **2**, 1172–1174.
27 Miner, J. and Hoffhines, A. (2007) The discovery of aspirin's antithrombotic effects. *Tex. Heart Inst. J.*, **34**, 179–186.
28 Wright, I.S. (1955) Present status of anticoagulant therapy in the treatment of myocardial infarction; the use and misuse of anticoagulants; an evaluation of new anticoagulants, their indications and dosage. *Ann. Intern. Med.*, **43** (5), 942–954.
29 Wright, I.S., Marple, C.M., and Beck, D.F. (1954) *Myocardial Infarction: Report of the Committee on Anticoagulants of the American Heart Association*, Grune & Stratton, Inc., New York.
30 Link, K.P. *et al.* (1943) Studies on the hemorrhagic sweet clover disease: XI. Hypoprothrombinemia in the rat induced by salicylic acid. *J. Biol. Chem.*, **147**, 463–473.
31 Link, K.P. (1959) The discovery of dicumarol and its sequels. *Circulation*, **19**, 97–107.
32 Craven, L.L. (1950) Acetylsalicylic acid, possible preventive of coronary thrombosis. *Ann. West Med. Surg.*, **4** (2), 95.
33 Craven, L.L. (1953) Experiences with aspirin (acetylsalicylic acid) in the non-specific prophylaxis of coronary thrombosis. *Miss. Valley Med. J.*, **75**, 38–40.
34 Craven, L.L. (1956) Prevention of coronary and cerebral thrombosis. *Miss. Valley Med. J.*, **78**, 213–215.
35 Dalen, J.E. (1991) An apple a day or an aspirin a day? *Arch. Intern. Med.*, **151** (6), 1066–1069.
36 Elwood, P.C. *et al.* (1974) A randomized controlled trial of acetyl salicylic acid in the secondary prevention of mortality from myocardial infarction. *Br. Med. J.*, **1** (5905), 436–440.
37 Reilly, I.A. and FitzGerald, G.A. (1988) Aspirin in cardiovascular disease. *Drugs*, **35** (2), 154–176.
38 US-PHS (1988) Findings from the aspirin component of the ongoing Physicians' Health Study. *N. Engl. J. Med.*, **318** (4), 262–264.
39 ISIS-2 (1988) Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. *Lancet*, **2** (8607), 349–360.
40 Antiplatelet Trialists, A. (1988) Antiplatelet Trialists' Collaboration: secondary prevention of vascular disease by prolonged antiplatelet treatment. *BMJ*, **269**, 320–331.

1.1.5 Current Research Topics

Aspirin, its pharmacology, and clinical applications are still subject of intense basic and clinical research. Over the last 10 years (2005–2014), the publication rate of papers with the entry "aspirin" alone in the scientific reference citation base PubMed amounted constantly to 2500 and more – every year. It will be interesting to see the impact of new discoveries regarding the pathophysiology of diseases, speicifically immunoloigical aspects of inflammation and, in particular new acetylation targets in the future.

1.1.5.1 Clinical Research

Two areas of clinical research are currently of particular interest for aspirin and both primarily for its preventive, long-term use: cardiovascular (primary) prevention and prevention of colorectal cancer. Both will be discussed below in more detail. Nevertheless, there are additional fields of clinical aspirin research that are less well known, although probably not less important. For example, (early) aspirin medication becomes increasingly of interest for prevention of preeclampsia in women at enhanced risk. Reasons for this consideration besides its efficacy and the lack of pharmacological alternatives was the good tolerance and the apparent absence of any significant toxicity to the fetus, even at the early start of treatment [1]. The identification of biomarkers, early defining women at elevated risk for the disease, will further help to improve the benefit–risk ratio. Another field of interest is neurological disorders, including cognitive deficits of Alzheimer's disease and their prevention by aspirin, although the evidence is sparse until now [2]. Finally, anti-inflammatory actions of low-dose aspirin are coming increasingly into focus, specifically the generation of aspirin-triggered lipoxin subsequent to acetylation of COX-2. However, antiplatelet effects via COX-1 inhibition might also be involved. Recent data suggest antiplatelet effects of aspirin as possibly important in early treatment of the "systemic inflammatory response syndrome" (SIRS), sepsis, and the respiratory distress syndrome [3–5] as well as platelet-triggered immunomodulatory actions of aspirin in HIV patients, which might contribute to prevention of the elevated infarct risk in these patients (Section 4.2.2) [6]. New information in these exciting fields of research will be expected in the near future [7].

Cardiovascular Prevention The available clinical data on aspirin in primary prevention of cardiovascular events are still under debate, specifically with respect to the benefit/risk (bleedings) ratio during long-term prophylactic use. Currently, the general opinion is that aspirin use for primary prevention in subjects at low cardiovascular risk (<1 event per year) is not justified (Level IIIB Recommendation of the European Heart Association, 2012). However, the benefit/risk ratio improves with increasing risk factors, such as hypertension, hypercholesterolemia, diabetes, and others. Unfortunately, there are only very few prospective randomized trials in medium-to-moderate risk patients. The prospective, randomized ARRIVE trial in patients at medium-elevated cardiovascular risk (expected annual event rate 2–3; no diabetics!) is underway and will probably be finished in 2016. In addition, the role of the platelet turnover rate as a variable for aspirin efficacy has long been underestimated. It has been assumed that "one aspirin a day" or even each other day fits all cases that need aspirin protection from thrombotic events. However, the platelet turnover rate may be increased and their survival shortened due to interactions between atherosclerotic vessel walls and platelets [8]. This might result in the appearance of significant amounts of "unblocked" platelets in the circulation at once-daily aspirin administration. As already seen in the first studies on aspirin and platelet function, about 10% untreated platelets may be sufficient to correct the aspirin-induced platelet "abnormalities" [9]. An increased platelet turnover exists in diabetics. In this population,

available trials on aspirin prevention of myocardial infarction and other vascular events (ETDRS, POPADAD, JPAD) did not provide convincing results (Section 4.1.1). One explanation for this might be an increased platelet turnover rate in these subjects [10–12]. The ASCEND and ACCEPT-D trials are underway to study this issue in more detail.

Colorectal Cancer First evidence for a chemopreventive action of aspirin in colorectal cancer was obtained in 1988 from the Melbourne Colorectal Cancer Study – the same year when the first two pioneering studies on primary (US-Physicians' Health) and secondary (ISIS-2) cardiovascular prevention appeared. *Gabriel Kune et al.* reported a 40% reduced risk of incident colon cancer among regular aspirin users compared to those who did not regularly take the drug. The results of the study were summarized by Dr. Kune as follows:

> *. . . There was a statistically significant deficit of the use of aspirin and aspirin-containing compounds among cases and these differences remained statistically significant after adjustment for hypertension, heart disease, chronic arthritis, and diet in both males and females . . . This finding, whatever the mechanism may be, has potential significance in colorectal cancer chemoprevention and merits early confirmation. Aspirin is now widely used in the chemoprophylaxis of cardiovascular disease and may also be useful in a similar way in the prevention of colorectal cancer and perhaps also of other cancers* [13].

These data were generated in a retrospective, exploratory case–control study that also noted a remarkable risk reduction in subjects using NSAIDs other than aspirin. Similar findings were also obtained in the Boston Collaborative Study [14]. More recently, *Peter M. Rothwell et al.* [15–17] have reinvestigated data from earlier cardiovascular prevention trials with aspirin. These studies have the advantage of a longer observation period, currently about 20 years, and a randomized design for the initial phase of the cardiovascular study. These observational data confirmed a time-dependently reduced incidence of colorectal cancer, which was comparable for both men and women. Interestingly, aspirin also reduced the risk of distal metastases of preexisting carcinomas, and there was a long-term survival benefit in some aspirin patients. This effect appeared to be correlated with particular genotypes and was due to nonvascular reasons but not due to a reduced rate of vascular events. At the same time, there was a marked and, again, time-dependent reduction of bleedings, including a 50% reduction in fatal cerebral bleeds. These exciting findings are from observational trials and need to be confirmed in prospective randomized studies with colorectal tumors as a clinical endpoint. Prospective randomized trials in primary and secondary prevention (Add-Aspirin-Trial; ASCOLT, JCAPP) are underway. Closely related to these studies is also the search for possible modes of action of aspirin as well as the evaluation of suitable biomarkers to identify those subgroups of patients who benefit most. Interestingly, there appears to be a clear benefit of aspirin after an appropriately long duration of treatment, that is, 5–10 years or more, in gastrointestinal cancers but possibly less in other carcinomas or malignancies [18]. The US Preventive Services Task Force has recently (September 2015) released a draft recommendation for the use of aspirin as a colorectal cancer preventive in selected groups of adults. This might broaden the use of aspirin in primary prevention (Section 4.3.1).

1.1.5.2 Basic Research

Aspirin "Resistance" One issue of interest in basic research with a considerable clinical relevance is the so-called aspirin "resistance," that is, a reduced antiplatelet effect of aspirin in certain clinical conditions. It is now becoming increasingly clear that an insufficient response to aspirin in almost every case is not due to a pharmacological failure of the drug to act, that is, to sufficiently block platelet COX-1, but rather due to clinical conditions of the patient. In addition, there are interactions with other drugs.

Negative impacts have been described in particular for several NSAIDs interacting with the aspirin binding site in the COX-1 substrate channel. This has been shown for ibuprofen and indomethacin, but also for dipyrone (metamizole), a non-anti-inflammatory analgesic [19]. All these compounds can markedly reduce the antiplatelet effect of aspirin [20]. Another issue is whether platelet function testing, that is, measurement of platelet aggregate formation *ex vivo*, provides sufficient information for the *in vivo* situation of inhibition of platelet function [21]. In this context, anti-inflammatory actions of aspirin, either by inhibition of release of inflammatory platelet-derived products or by interaction with other cells (white cells, endothelium) come increasingly into focus [22,23] and will not be detected by conventional measurement of platelet aggregation *ex vivo*. Platelets are also considered an important part of the innate immune system. Thus, platelets are multipurpose cells in the interplay between hemostasis, thrombosis, inflammation, and cancer and targeted inhibition of platelet functions by aspirin might be of much greater significance for processes beyond aggregate formation than currently appreciated.

Aspirin and COX-2-Derived Products A particular pharmacological property of aspirin that is not shared with traditional NSAIDs or coxibs is the acetylation of COX-2 with subsequent generation of 15-(*R*)-HETE and aspirin-triggered lipoxin, an *anti*-inflammatory and inflammation-resolving mediator, by intercellular interactions with other lipoxygenases. This may help to better understand and explain clinically well-known phenomena, such as adaptation of stomach mucosa to long-term (high-dose) aspirin use (Section 3.2.1) [24] and the inhibition of white cell recruitment to an inflamed site [25,26] (Section 2.3.2). In addition, COX-2 has a broad substrate specificity, also including neutral lipids and endocannabinoids. Their generation and action may also become modified after COX-2 acetylation by aspirin, but is insufficiently studied yet. These activities might be relevant to all clinical situations with an upregulated COX-2, including acute and chronic inflammation, tumorigenesis, and advanced atherosclerosis.

Acetylation of Further Molecular Targets by Aspirin Prostaglandins are the key mediators of interest to understand the biological effects of aspirin and COX inhibition by acetylation is the mode of action of aspirin involved. However, these are not the only cellular acetylation targets. For example, the successful use of aspirin in treatment of headache and migraine obviously involves actions on other pain mediators, such as serotonin or endocannabinoids (anandamide), that are poorly understood yet. Another mediator system of interest is endothelial nitric oxide, made by the endothelial NO synthase. Aspirin stimulates endothelial NO formation by posttranslational lysine acetylation of this enzyme [27], eventually resulting in activation of heme oxygenase-1 and improved endothelial protection. Recent proteomic studies have identified >100 peptide/protein sequences that become (irreversibly) acetylated by aspirin *in vitro* [28,29]. The lowest aspirin concentrations for detectable protein acetylation were in the range of 50–100 μM [28]. Another recent mapping site analysis has identified more than 500 potential aspirin-modified proteins with high confidence, the majority of them being lysine acetylated [30] (Figure 1.1-5-1). The biological consequences of these (irreversible) acetylations are still unknown but might help us to understand long lasting effects of aspirin – the half-life for human albumin amounts to 19 days! It can, therefore, not be excluded that regular low-dose daily aspirin results in an accumulation of acetylated structures, such as lysines, eventually also resulting in sufficient modification of protein functions that require 100 μM or higher concentrations at single application.

Aspirin Analogues Finally, the discovery of new and real "aspirin-like" drugs, that is, drugs that irreversibly acetylate molecular targets, is a

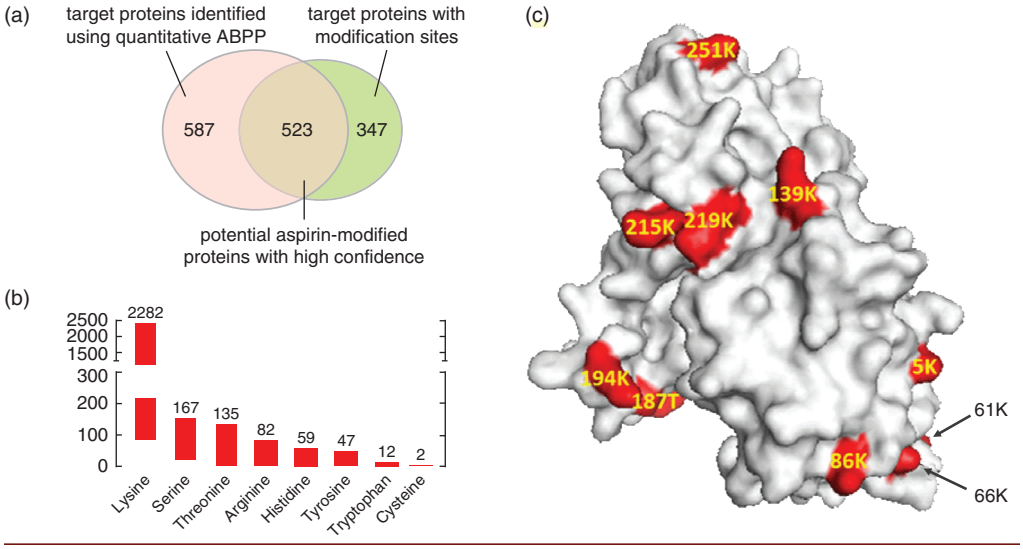


Figure 1.1-5-1 Mapping sites of aspirin-induced acetylations in live cells by quantitative acid-cleavable activity-based protein profiling (QA-ABPP). (Modified after Ref. [30]).

pharmacological challenge and, eventually, might result in design and development of new class(es) of anti-inflammatory, anticancer, and pain-relieving drugs. One such aspirin analogue that binds covalently and irreversibly to COX-2 (*o*-[acetoxy-phenyl]hept-2-ynyl sulfide, APHS), is already available [31]. It was found to be equipotent to aspirin in stimulating endothelial NO production *in vitro* [32] and to have aspirin-like antitumor effects in certain experimental settings [33]. The recent elucidation of the crystal structure of aspirin-acetylated COX-2 [34] will certainly further stimulate the design and development of new "aspirin-like" drugs

Table 1.1-1 The history of salicylates and acetylsalicylic acid.

Date	Event
400 BC–100 AC	*Hippocrates* recommends bark and leaves of the willow tree (*salix alba*) for medical use. This recommendation is later compiled by *Pliny* and *Dioscurides* as popular medical knowledge of the time
1763	Rev. *Edward Stone* recommends the use of willow bark extracts for treatment of "Aigues and intermitting disorders"
1826–1830	*Brugnatelli* and *Fontana* as well as *Buchner* identify salicin as the active antipyretic ingredient of the willow bark. *Leroux* in 1830 is the first to isolate salicin in crystalline form. The compound, prepared from willow bark, is later sold by *Ernst Merck* as an antipyretic drug for half the prize of quinine
1839	*Piria* prepares salicylic acid from salicin and correctly determines the brutto formula $C_7H_6O_3$
1835–1843	New, rich sources of natural salicylates are detected, most notably wintergreen oil from the American Evergreen (*Gaultheria procumbens*), containing about 99% methylsalicylate. This finding markedly improves the availability of salicylates for practical use

(*continued*)

Table 1.1-1 (*Continued*)

Date	Event
1859–1874	*Hermann Kolbe* synthesizes for the first time pure salicylic acid from the already known decomposition products phenol and carbonic acid. His assistant *Rudolf Wilhelm Schmitt* and his student *Friedrich von Heyden* improve the technology of synthesis. Von Heyden founded the first salicylic acid-producing factory in Radebeul (near Dresden) in 1874. The plant rapidly produced tons of salicylic acid. This provides unlimited amounts of the compound for medical use, and independence of natural sources
1875	*Ebstein* and *Müller* detect the blood sugar-lowering action of salicylates
1876	*Stricker* introduces salicylate as an analgesic/antirheumatic drug at the Charité in Berlin. Shortly thereafter, *MacLagan*, a Scottish physician, and *Germain Sée*, a Frenchman, from Straßburg (Alsace) also describe an antipyretic/analgesic activity of the compound
1897	*Felix Hoffmann*, working in the pharmaceutical research group at Bayer laboratories in Elberfeld under direction of *Arthur Eichengrün*, synthesizes for the first time acetylsalicylic acid as a chemically pure and stable compound
1899	*Heinrich Dreser*, head of the pharmacological research laboratories at Bayer, publishes the first report on the pharmacology of acetylsalicylic acid. He considers the compound as a prodrug of the active metabolite salicylic acid with the advantage of a better taste and less toxicity to the stomach. The first clinical studies by *Kurt Witthauer* and *Julius Wolgemuth* are published the same year
1899	Introduction of acetylsalicylic acid to the market for the treatment of fever and pain under the trade name "Aspirin®"
Since 1899	Worldwide use of Aspirin as a household remedy for treatment of fever, pain, and inflammation
1902	First description of a hypersensitivity reaction (dyspnea, urticaria, angioedema) to aspirin by *G. Hirschberg* from Posen.
1945–1952	*Singer* describes a bleeding tendency after surgical interventions if aspirin was used for analgesic purposes. This observation is confirmed in other case reports. Singer explains this by a reduction of prothrombin levels
1949–1950	*Gibson* reports on the positive results with aspirin for treatment of angina pain according to a survey of 20 physicians. He ascribes this effect to a combination of analgesic and antithrombotic activities of aspirin, the last being similar to coumarins from which salicylate is formed as a metabolic intermediate
1950–1956	*Lawrence L. Craven*, a General Practitioner from Glendale (California), publishes a series of studies using aspirin as "coumarin-light" for prevention of myocardial infarction and stroke. According to the data from his first study, daily administration of 650–1950 mg aspirin completely prevented myocardial infarctions in 400 medium-aged male patients during an observation period of 2 years. In the following years, he increases the number of patients up to about 8000 – reportedly without having seen any myocardial infarction – and finally reduces the dose to one tablet (1 tablet = 5 grain = 325 mg aspirin) per day, which is still a frequently used antiplatelet dose in the United States
1967–1968	*Breddin*, *O'Brien*, *Zucker*, *Weiss*, *Mustard*, and colleagues publish the first mechanistic studies on the antiplatelet activity of aspirin. Daily doses of 150 mg are sufficient to inhibit platelet function over several days. According to his data, O'Brien recommends a clinical trial with aspirin for thrombosis prevention in patients at elevated vascular risk
1969	The successful Apollo-11 mission to the moon has aspirin on board
1971	*Sir John Vane* detects the inhibition of prostaglandin synthesis by aspirin (and salicylate) and considers this as the mechanism of its anti-inflammatory and antipyretic action. This work and the later detection of prostacyclin are acknowledged with the Nobel Prize for Medicine in 1982.
1971	*Brian Smith* and *Al Willis*, both working in John Vane's laboratory, detect the inhibition of prostaglandin synthesis by aspirin in platelets. However, they do not see inhibition of (thrombin-induced) platelet function (secretion) by aspirin
1974	*Peter C. Elwood, Archibald L. Cochrane* and colleagues from Cardiff (Wales) publish the first randomized placebo-controlled on aspirin (300 mg/day) in men with recent myocardial infarction. They found a 25% reduced mortality after 1 year which, however, was not significant and consequently considered the data "inconclusive".

Table 1.1-1 (*Continued*)

Date	Event
1975	*Andrzej Szczeklik* and his group from Kraków (Poland) detect that precipitation of attacks in asthmatic patients by aspirin and related compounds is due to inhibition of prostaglandin biosynthesis
1975	*Philip Majerus* and *Jerry Roth* detect the irreversible acetylation of platelet cyclooxygenase by aspirin and explain by this mechanism the inhibition of thromboxane formation and platelet function. Later this laboratory identifies serine $_{530}$ as the molecular target of acetylation.
1979	*Philip Majerus* and coworkers also publish the first double-blind, randomized, placebo-controlled clinical trial on antithrombotic effects of an "antiplatelet dose" (160 mg/day) aspirin. Aspirin treatment reduces shunt thrombosis in hemodialysis patients within an observation period of 1 month highly significantly by 65%.
1979	*Crandon* and *Isherwood* report that regular intake of aspirin in pregnancy reduces the risk of preeclampsia. *Beaufils* confirms this finding in a randomized study with high-risk patients, published in 1985
1983	Publication of the first placebo-controlled, randomized, double-blind trial (Veterans Administration Study) on prophylactic use of aspirin (324 mg/day) in men with acute coronary syndromes by *Lewis* and colleagues. The study shows a 50% reduction of the incidence of myocardial infarctions and death within an observation period of 3 months
1988	Publication of the first study on a relationship between aspirin intake and prevention of colon cancer by *Gabriel Kune* and colleagues from Melbourne (Australia). In this retrospective, exploratory case-control study, regular (daily) use of aspirin reduced the risk of (incident) colon cancer by 40%. These findings were later confirmed and extended by many others in a large epidemiological and some randomized clinical trials.
1988–1989	Publication of the first two prospective long-term trials on primary prevention of myocardial infarctions in apparently healthy men in the United States (USPHS) and United Kingdom (BMDS), respectively. The results are controversial. The placebo-controlled American study suggested a beneficial effect of aspirin on the prevention of a first myocardial infarction, which was not seen in the open British trial. *Charles Hennekens* receives the Aspirin Senior Award in 1999 for his significant contributions to the use of aspirin for prevention of atherothrombotic events
1988	The ISIS-2 trial, a prospective, placebo-controlled, randomized trial in patients with acute myocardial infarction, demonstrates a 23% reduction in mortality by aspirin (162 mg/day) alone and a 38% reduction in combination with streptokinase for an observation period of 5 weeks. This study resulted in guideline recommendations of aspirin for secondary prevention of myocardial infarction
1988–1990	*William Smith*, *David De Witt*, and colleagues demonstrate that the molecular mechanism of action of aspirin is steric hindering of the access of substrate (arachidonic acid) to the enzyme (cyclooxygenase) and does not involve direct binding of the agent to the active center. *William Smith* receives the Aspirin Senior Award in 1997 for this and other major contributions elucidating the molecular mechanism of aspirin action
1991	Publication of the first prospective, randomized, placebo-controlled study (SALT Trial) on low-dose (75 mg/day) aspirin in patients with transient ischemic attacks (TIA). The number of strokes, TIA, and myocardial infarctions is markedly reduced by aspirin, while the number of hemorrhagic cerebral infarctions is increased. The benefit/risk ratio is clearly in favor of prevention
1991	*Kenneth K. Wu* and his group show inhibition of cytokine-induced expression of cyclooxygenase-2 in human endothelial cells by aspirin and salicylate but not by indomethacin. This suggests salicylate-mediated inhibition of COX gene transcription. Later work of his group identifies inhibition of binding of the C/EBPβ transcription factor as (one) molecular mechanism of action

(*continued*)

Table 1.1-1 (*Continued*)

Date	Event
1995	*Patrick Loll*, *Daniel Picot*, and *R. Michael Garavito* describe the crystal structure of COX-1 inactivated by an aspirin analogue. Patrick Loll receives the Aspirin Junior Award in 2000 for his significant contributions to this work
1995	*Joan Claria* and *Charles Serhan* detect the generation of aspirin-triggered lipoxin (ATL) by the interaction of acetylated COX-2 with the 5-lipoxygenase of white cells. The contributions of Claria, working in Serhan's group, to this research are acknowledged with the Young Researchers' Aspirin Award in 1996
2000	The Oxford Group of *Sir Richard Peto*, *Rory Collins*, and *Peter Sleight* receives the Aspirin Senior Award for their outstanding activities in developing and performing meta-analyses on studies with antiplatelet agents. Data elaborated with this technology by the "Antiplatelet (antithrombotic) Trialists' Collaboration" show an overall >20% risk reduction of new severe vascular events in secondary prevention by regular aspirin, however with wide variations between different risk groups and a positive benefit/risk ratio despite increased bleeding
2005	Publication of the prospective, randomized, placebo-controlled *W*omen's *H*ealth *S*tudy (WHS). The study demonstrates modest but significant protection from ischemic cerebral infarctions but not from myocardial infarctions by aspirin (100 mg each second day) in apparently healthy women during a 10 year observation period.
2006	The CHARISMA study compares low-dose aspirin (75–162 mg) alone and in combination with clopidogrel (75 mg) for primary prevention of vascular events in high-risk populations. While the combination is useful in secondary prevention, the comedication of clopidogrel with aspirin did not reduce the vascular risk but significantly increased bleeding in subjects with risk factors but without a preexisting event
2011/2012	*Peter W. Rothwell* and colleagues publish a series of articles (metaanalyses) on the chemopreventive effect of aspirin in primary and secondary prevention of colorectal cancer. Important data on long-term effects were generated from earlier trials on cardiovascular prevention, which were reinvestigated after an about 15–20 year's observation period. The three main findings for aspirin were as follows: (i) a significant reduction in cancer mortality in both primary and secondary prevention, which accounts for the long-term survival benefit in these patients rather than cardiovascular prevention, (ii) the increased incidence of severe and/or life-threatening bleeding becomes markedly (about 50%) reduced with time, and (iii) these beneficial effects of aspirin are seen at antiplatelet doses of around 100 mg/day and appear not to become stronger with increasing doses
2014	*Leslie A. Bateman* and colleagues identify 112 new proteins that are irreversibly (lysine) acetylated by aspirin, threshold aspirin concentrations are in the range of 50–100 μM. At least some of them might be relevant to cell proliferation and energy metabolism
2014	A new fast disintegrating oral aspirin formulation with a threefold higher peak plasma level of unmetabolized aspirin and a more than twice as much faster onset of action is introduced to the market.
2015-	Intense basic and clinical research tries to further identify cellular targets of aspirin to clarify aspirin's mode of chemopreventive action and to study the relevance of these findings in prospective, randomized trials. In particular, focus is on biomarkers that could help identify individuals at elevated risk for cancer and preeclampsia
2015	The US Preventive Services Task Force (USPSTF) in a draft finds "adequate evidence" that aspirin use reduces the incidence of colorectal cancer in certain groups of adults after 10 years of use
2016	*Michael J. Lucido* and coworkers from *Michael G. Malkowski*'s group publish for the first time the crystal structure of aspirin-acetylated human COX-2

Summary

Current research areas of aspirin cover both basic and clinical research. A major topic in clinical research is the usefulness of aspirin as a chemopreventive in colorectal and perhaps other forms of cancer. In addition, studies are underway on aspirin for primary prevention of cardiovascular diseases, that is, myocardial infarction, in subjects at medium-to-moderate elevated vascular risk. This includes stable angina pectoris and the presence of certain risk factors, specifically diabetes. The possible benefits of aspirin in prevention of neurological disorders, specifically cognitive deficits, are also currently being investigated.

In basic research, aspirin "resistance," that is, the clinical failure of the drug to exert its antiplatelet effects despite sufficient inhibition of platelet COX-1 – the molecular target, is still a topic of interest, specifically the best methods for its measurement and possible biomarkers. Another topic is the aspirin-induced modulation of COX-2 activity, eventually resulting in the generation of new, anti-inflammatory compounds, such as lipoxins and others. There is also evidence for transacetylation of >100 proteins by aspirin *in vitro* with yet largely unknown biological consequences.

Finally, by analogy with other drugs, such as penicillin or ß-blockers, aspirin analogues may be developed with more cell-specific action profiles. One compound that selectively and covalently binds to COX-2 has already been designed. It was found *in vitro* to stimulate endothelial NO production and to have antitumor effects. Others may follow, eventually resulting in the design and development of new and even more effective class(es) of "aspirin-like" drugs.

References

1 Henderson, J.T., O'Connor, E., and Whitlock, E.P. (2014) Low-dose aspirin for prevention of morbidity and mortality from preeclampsia. *Ann. Intern. Med.*, **161** (8), 613–614.

2 Wang, J. *et al.* (2015) Anti-inflammatory drugs and risk of Alzheimer's disease: an updated systematic review and meta-analysis. *J. Alzheimers Dis.*, **44** (2), 385–396.

3 Akinosoglou, K. and Alexopoulos, D. (2014) Use of antiplatelet agents in sepsis: a glimpse into the future. *Thromb. Res.*, **133** (2), 131–138.

4 O'Neal, H.R.J., Koyama, T., Koehler, E.A. *et al.* (2011) Prehospital statin and aspirin use and the prevalence of severe sepsis and acute lung injury/acute respiratory distress syndrome. *Crit. Care Med.*, **39** (6), 1343–1359.

5 Lösche, W., Boettel, J., Kabisch, B. *et al.* (2012) Do aspirin and other antiplatelet drugs reduce the mortality in critically ill patients? *Thrombosis.*, **2012**, 720254.

6 O'Brien, M. *et al.* (2013) Aspirin attenuates platelet activation and immune activation in HIV-1-infected subjects on antiretroviral therapy: a pilot study. *J. Acquir. Immune Defic. Syndr.*, **63** (3), 280–288.

7 Berk, M. *et al.* (2013) Aspirin: a review of its neurobiological properties and therapeutic potential for mental illness. *BMC Med.*, **11**, 74.

8 Ritchie, J.L. and Harker, L.A. (1977) Platelet and fibrinogen survival in coronary atherosclerosis: response to medical and surgical therapy. *Am. J. Cardiol.*, **39** (4), 595–598.

9 O'Brien, J.R. (1968) Effects of salicylates on human platelets. *Lancet*, **1**, 779–783.

10 Dillinger, J.G. *et al.* (2012) Biological efficacy of twice daily aspirin in type 2 diabetic patients with coronary artery disease. *Am. Heart J.*, **164** (4), 600–606.

11 Rocca, B. *et al.* (2012) The recovery of platelet cyclooxygenase activity explains interindividual variability in responsiveness to low-dose aspirin in patients with and without diabetes. *J. Thromb. Haemost.*, **10** (7), 1220–1230.

12 Capodanno, D. *et al.* (2011) Pharmacodynamic effects of different aspirin dosing regimens in type 2 diabetes mellitus patients with coronary artery disease. *Circ. Cardiovasc. Interv.*, **4** (2), 180–187.

13 Kune, G.A., Kune, S., and Watson, L.F. (1988) Colorectal cancer risk, chronic illnesses, operations, and medications: case control results from the

Melbourne Colorectal Cancer Study. *Cancer Res.*, **48** (15), 4399–4404.
14 Rosenberg, L. *et al.* (1991) A hypothesis: nonsteroidal anti-inflammatory drugs reduce the incidence of large-bowel cancer. *J. Natl. Cancer Inst.*, **83** (5), 355–358.
15 Rothwell, P.M. *et al.* (2012) Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet*, **379** (9826), 1602–1612.
16 Rothwell, P.M. *et al.* (2012) Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet*, **379** (9826), 1591–1601.
17 Rothwell, P.M. *et al.* (2012) Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet*, **379** (9826), 1602–1612.
18 Cuzick, J. *et al.* (2015) Estimates of benefits and harms of prophylactic use of aspirin in the general population. *Ann. Oncol.*, **26** (1), 47–57.
19 Hohlfeld, T. and Schrör, K. (2015) Inhibition of antiplatelet effects of aspirin by non-opioid analgesics. *Clin. Pharmacol. Ther.*, **97** (2), 131–134.
20 Hohlfeld, T., Saxena, A., and Schrör, K. (2013) High on treatment platelet reactivity against aspirin by non-steroidal anti-inflammatory drugs: pharmacological mechanisms and clinical relevance. *Thromb. Haemost.*, **109** (5), 825–833.
21 Schrör, K., Huber, K., and Hohlfeld, T. (2011) Functional testing methods for the antiplatelet effects of aspirin. *Biomark. Med.*, **5** (1), 31–42.
22 Hohlfeld, T. and Schrör, K. (2015) Antiinflammatory effects of aspirin in ACS: relevant to its cardiocoronary actions? *Thromb. Haemost.*, **114**, 469–477.
23 Schrottmaier, W., Kral, J., Badrnya, S., and Assinger, A. (2015) Aspirin and P2Y12 inhibitors in platelet-mediated activation of neutrophils and monocytes. *Thromb. Haemost.*, **114**, 478–489.
24 Fiorucci, S. *et al.* (2002) Cyclooxygenase-2-derived lipoxin A4 increases gastric resistance to aspirin-induced damage. *Gastroenterology*, **123** (5), 1598–1606.
25 Claria, J. and Serhan, C.N. (1995) Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell–leukocyte interactions. *Proc. Natl. Acad. Sci. USA*, **92** (21), 9475–9479.
26 Morris, T. *et al.* (2009) Effects of low-dose aspirin on acute inflammatory responses in humans. *J. Immunol.*, **183** (3), 2089–2096.
27 Jung, S.B. *et al.* (2010) Histone deacetylase 3 antagonizes aspirin-stimulated endothelial nitric oxide production by reversing aspirin-induced lysine acetylation of endothelial nitric oxide synthase. *Circ. Res.*, **107** (7), 877–887.
28 Bateman, L.A. *et al.* (2013) An alkyne-aspirin chemical reporter for the detection of aspirin-dependent protein modification in living cells. *J. Am. Chem. Soc.*, **135** (39), 14568–14573.
29 Marimuthu, S. *et al.* (2011) Aspirin acetylates multiple cellular proteins in HCT-116 colon cancer cells: identification of novel targets. *Int. J. Oncol.*, **39** (5), 1273–1283.
30 Wang, J. *et al.* (2015) Mapping sites of aspirin-induced acetylations in live cells by quantitative acid-cleavable activity-based protein profiling (QA-ABPP). *Sci. Rep.*, **5**, 7896.
31 Kalgutkar, A.S. *et al.* (1998) Aspirin-like molecules that covalently inactivate cyclooxygenase-2. *Science*, **280** (5367), 1268–1270.
32 Taubert, D. *et al.* (2004) Aspirin induces nitric oxide release from vascular endothelium: a novel mechanism of action. *Br. J. Pharmacol.*, **143** (1), 159–165.
33 Humar, B. *et al.* (2008) Heterogeneous gene expression changes in colorectal cancer cells share the WNT pathway in response to growth suppression by APHS-mediated COX-2 inhibition. *Biologics*, **2** (2), 329–337.
34 Lucido, M.J. *et al.* (2016) The crystal structure of aspirin acetylated human cyclooxygenase-2: insight into the formation of products with reversed stereochemistry. *Biochemistry*. doi: 10.1021/acs.biochem.5b01378

1.2 Chemistry

This chapter is focused on pharmaceutical aspects of aspirin and selected salicylates. This includes physicochemical properties of the compounds as well as methods for determination of aspirin and its metabolites. This section is not written with the intention to provide a complete overview of all pharmaceutically relevant aspects of salicylates but rather to inform about those that are particular relevant to the understanding of their pharmacology and toxicology in biological systems.

Chemical structures and physicochemical properties of aspirin and other salicylates are dealt with in the first part (Section 1.2.1). In this context, the unique mesomeric structure of salicylate, allowing its incorporation and enrichment in cell membrane phospholipids, is of outstanding significance to understand the (nonspecific) actions of salicylic acid on cellular energy metabolism, specifically the uncoupling of oxidative phosphorylation. Another aspect is the (poor) water solubility of aspirin in acidic media, such as stomach juice, eventually resulting in local tissue irritation but also rapid passage of the compound into the small intestine. Finally, the particular crystal structure of aspirin and the recent discovery of two polymorphic forms, coexisting in one and the same aspirin crystal, are of considerable pharmacological interest.

The second part of this chapter describes analytical methods of salicylate determination in biological media (Section 1.2.2). Several techniques are available, allowing simultaneous measurement of both aspirin and its major metabolites in biological samples at low micromolar to nanomolar concentrations. These concentrations are to be expected after antiplatelet and analgesic/anti-inflammatory doses *in vivo*.

1.2.1 Structures and Chemical Properties of Salicylates

1.2.1.1 Salicin: The Natural Salicylate

The ß-glucoside salicin with the aglycon saligenin is the natural pharmacologically active ingredient of willow bark, isolated for the first time in crystalline form by Leroux in 1830 (Section 1.1.1). Leroux obtained 1 oz (about 28.3 g) of salicin from 3 lb of willow bark (*Salix helix*) (cited after Ref. [1]). Salicin was later used by Piria as starting material for preparation of salicylic acid (Section 1.1.1). Salicylic acid (*o*-monohydroxy benzoic acid) is a relatively strong acid with a pK_A of 2.9 and is poorly water soluble (0.2%). The solubility can be considerably increased by conversion into the sodium salt, which is approximately 50% water soluble. Salicylates for systemic use are either esters with substitutions at the carboxyl group, such as methylsalicylate, or esters of organic acids with substitutions in the phenolic *o*-hydroxyl group, such as aspirin. Aspirin is the acetate ester of salicylic acid (Figure 1.2.1-1). The crystalline and molecular structure of aspirin has been elucidated [2,3].

Computer calculations have suggested another even more stable crystalline isoform of aspirin in addition to the already known isoform I. Experimental studies were able to confirm the real existence of the isoforms I and II and, in addition, also demonstrated a polymorphism between these two isoforms. Importantly, the two different polymorphs can coexist within one and the same crystal [4,5]. *This new and unexpected finding with aspirin as the first compound to show this unique property raises a number of principal physicochemical questions regarding definition of crystal polymorphism. A detailed evaluation of this is outside the requirements for understanding the pharmacological and toxicological properties of the compound. However, there are also legal issues that might be important for drug development – each polymorph of each compound can be patented separately.*

1.2.1.2 Salicylates in Clinical Use

Salicylic Acid Salicylic acid (molecular weight 138.1) in the form of sodium salt (molecular weight 160.1) was the first entirely synthetic salicylate for medical use (Section 1.1.1). It is now no longer used for internal applications. One reason for this is the unpleasant, sweaty-bitter taste caused by direct stimulation of the human bitter taste receptor [6]. More importantly, salicylate is a direct irritant of the stomach mucosa. Historically, this was the major cause for search for better

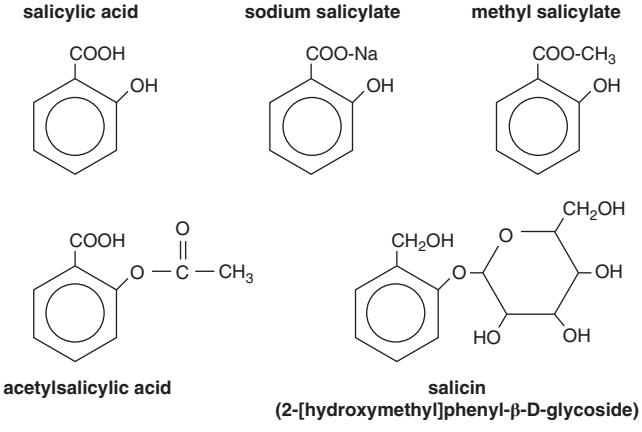


Figure 1.2.1-1 Chemical structure of selected salicylates.

tolerable derivatives, such as the acetylated product – acetyl salicylate (aspirin), which was reportedly salicylate-"free" [7] and has replaced salicylate as a medicine for internal use. However, salicylate is still being used as an external medication, for example, in ointments, because of its antiseptic and keratolytic properties (corn plaster).

Despite its disappearance from internal use, the pharmaceutical and biological properties of salicylate are still of considerable pharmacological interest. Salicylate is the primary metabolite of aspirin and responsible for many of its biological actions on cell function, including the symptoms of salicylate poisoning (Section 3.1.1). Salicylate shows a peculiar physicochemical behavior because of the formation of a ring structure by hydrogen bridging. This requires a hydroxy group in a close neighborhood to the carboxyl group and is only seen with the *o*-hydroxy benzoic acid salicylic acid (Figure 1.2.1-2), but not with its *m*- and *p*-analogues. The *o*-position of the hydroxyl group facilitates the release of a proton with decreasing pH by increasing the mesomery of the resulting anion. These properties explain the protonophoric actions of salicylates in uncoupling of oxidative phosphorylation by eliminating the impermeability of mitochondrial cell membranes for protons [8] (Section 2.2.3). In addition, they help understand the local irritation of the stomach mucosa as a consequence of direct contacts and subsequent pH-dependent uptake into mucosal cells (Section 3.2.1). The *m*- and *p*-hydroxy analogues of benzoic acid do not share these properties with salicylate and are biologically inert (Figure 2.3.2-3).

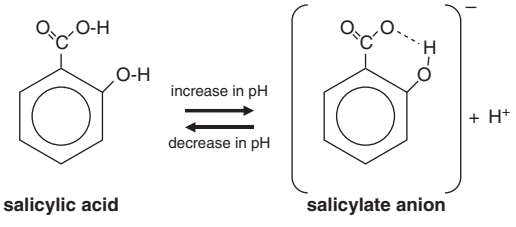


Figure 1.2.1-2 pH-dependent equilibrium of ionized and nonionized forms of salicylate.

Structure–activity comparisons were made with 80 salicylate analogues in order to clarify the relationship between chemical structure and uncoupling of oxidative phosphorylation. Studies in isolated mitochondria showed that the essential pharmacophore for this activity is a compound with a negatively charged (carboxyl) group at the o-position, that is, acetylsalicylate. The m- and p-hydroxy benzoate analogues of salicylic acid have no effect. This suggested the o-position of the hydroxyl group as an essential steric requirement for its metabolic activity. Mechanistically, this was explained by the unique proton bridging between the oxygen of the carboxyl group and the adjacent proton in the hydroxyl group. This allowed a mesomeric state that promoted a nondissociated configuration and facilitated tissue penetration [9].

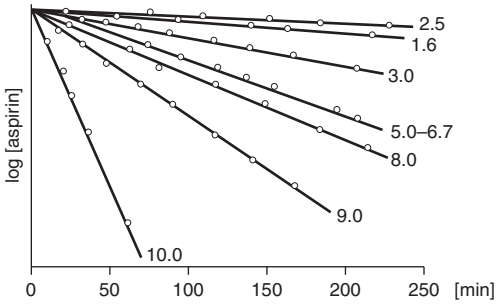


Figure 1.2.1-3 pH-dependent hydrolysis of aspirin (1.5 mM) in aqueous solution at 42 °C. Note the high stability (poor solubility) of aspirin at acidic pH and the significant increase in solubility at increasing alkaline pH [10].

Acetylsalicylic Acid Acetylsalicylic acid (molecular weight 180.2) or aspirin is the acetate ester of salicylic acid. The pharmacological properties are similar to those of salicylate. However, aspirin has additional activities of its own that are added by the reactive acetate group – the (nonselective) acetylation of cellular targets, including proteins and DNA. This results in biological effects that are not shared by salicylate. Examples are the inhibition of platelet function by irreversible acetylation of COX-1 and the generation of aspirin-triggered lipoxin by acetylation of COX-2, with subsequent formation of 15-(*R*)-HETE and generation of ATL by white cell 5-lipoxygenases (Section 2.2.1).

Aspirin is a white powder with a sour taste. The compound is poorly soluble in water (0.3%) but somewhat better in ethanol (20%). The solubility in aqueous media is pH dependent. It amounts to only 60 µg/ml at pH 2 but increases dramatically with increasing pH (Figure 1.2.1-3) [10]. The solubility in aqueous media is also markedly improved after conversion into the sodium salt, specifically at acidic pH. The pH-dependent marked changes in solubility of aspirin are the reason for its largely unchanged and rapid passage of the acidic gastric juice (Section 3.2.1) as well as the rapid absorption of the dissolved compound inside the upper intestine.

Methylsalicylate Methylsalicylate is the active ingredient of wintergreen oil from the American Teaberry (*Gaultheria procumbens)*. This oil was used as a natural source of salicylates since the early nineteenth century because of its rich content of methylsalicylate. Methylsalicylate is the active constituent of numerous drug combinations for external use, for example, rheuma ointments and bath salts. It is also of toxicological interest because of its much higher toxicity – as opposed to other salicylates. Particularly dangerous is the erroneous ingestion (by children!) of methylsalicylate-containing ointments and other products for external use (Section 3.1.1).

1.2.1.3 Aspirin Formulations

Several galenic preparations of aspirin have been developed for practical use with the intention to improve the solubility and gastric tolerance of the compound. Rapid dissolution is particularly relevant if short-term or immediate action of aspirin is desired, for example, in treatment of migraine- or tension-type headache. Alternatively, aspirin can be administered intravenously or orally as the good water-soluble lysine salt. This is of particular interest if high levels of unmetabolized aspirin have to be obtained rapidly in the circulating blood, for example, to rapidly block platelet function in acute coronary syndromes (Section 4.1.1). Another approach is an enteric-coated formulation, predominantly for long-term use. The last development is a novel micronized, fast-disintegrating aspirin formulation with a faster and more complete systemic absorption compared to standard aspirin tablets [11] (Figure 1.2.1-4). A detailed discussion of the pharmacokinetics of these and other formulations in clinical use is found elsewhere (Section 2.1.1).

Generics A frequently discussed issue in clinical pharmacology is whether all formulations of all manufacturers containing the same amount of the active ingredient at the same dose are bioequivalent with the consequence to use preferentially the cheapest formulation. In case of

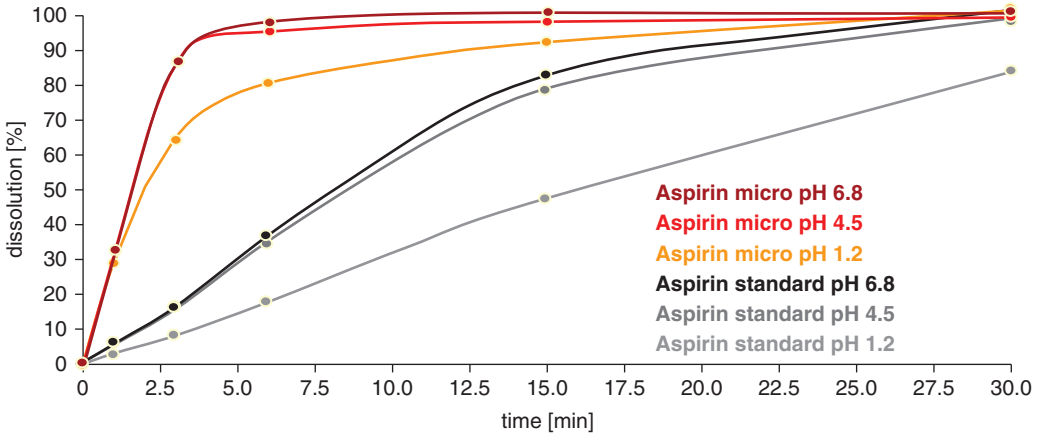


Figure 1.2.1-4 *In vitro* dissolution versus time curve of micronized (micro), fast disintegrating aspirin (500 mg tablet) and plain standard aspirin (500 mg tablet) at pH 1.2, 4.5, and 6.8. Note the much faster and largely pH-independent dissolution of the fast disintegrating form [11].

salicylates, this has been refused already many years ago [10]. A more recent study additionally suggested that even aspirin analogues that passed required *in vitro* dissolution specifications may not be bioequivalent *in vivo* [12]. Moreover, a comparative study of selected aspirin formulations in Germany has found large differences regarding the pharmaceutical quality of

Table 1.2.1-1 In vitro release kinetics of acetylsalicylic acid (ASA) under standard conditions from different commercial aspirin preparations in Germany.

ASA preparation	Declared ASA content (mg)	% of Declaration found	ASA release kinetics (% in 30 min ± SD)
Acetylin	500	98.9	89.6 ± 2.8
Aspirin	500	99.4	100.7 ± 1.0
Aspirin junior	100	102.9	103.5 ± 2.7
Aspro	320	98.1	96.4 ± 4.7
Ass 500 Dolormin	500	98.0	77.2 ± 9.2
ASS-Dura			
Ch.-B. 18613	500	98.2	65.4 ± 16.5
Ch.-B. 074035	500	102.9	72.4 ± 9.5
ASS-Fridetten			
CH.-B. 019026	500	98.3	68.8 ± 10.0
Ch.-B. 020047	500	100.1	75.9 ± 5.7
ASS-ratiopharm	500	96.6	89.3 ± 9.3
ASS-Woelm	500	100.9	77.9 ± 8.8
Temagin ASS 600			
Ch.-B. 212142	600	100.9	50.2 ± 11.4
Ch.-B. 212143	600	100.4	44.0 ± 9.9
Trineral	600	99.2	90.8 ± 3.9

13) According to the predefined quality standards, the content of the active ingredient should be 95–105% of declaration and at least 80% of the compound should be released within 30 min under the conditions chosen [13].

aspirin-containing monopreparations with suggested use as antipyretic analgesics.

In 1986, a pharmaceutical comparison of all aspirin OTC formulations on the German market was performed. Included were all products containing only aspirin as the active ingredient for suggested use as an antipyretic analgesic. Only tablets were included, but no other galenic preparations or tablets for other indications.

*All 11 brands fulfilled general pharmaceutical quality requirements, such as the content of the active ingredient. However, marked, and according to the authors, unacceptable differences existed with respect to the individual in vitro release kinetics. These criteria were determined according to an US standard and were not met by 5 out of the 11 preparations tested (*Table 1.2.1-1*)* [13].

The authors repeated this study 2 years later on 62 different aspirin preparations. They found that 20 (!) of the tested formulations still did not meet the quality standards mentioned above [14]. Thus, not all aspirin preparations might be the same in terms of bioequivalence *in vivo*, even if they contain the active ingredient (acetylsalicylic acid) in identical amounts.

Quality assessment of generics or similars might be a special problem in developing and threshold countries, where the income is low and OTC compounds are sold in all kinds of supermarkets and discounters without any quality control. A recent study in Brazil showed that from five tested brands, only genuine Bayer aspirin did fulfill the required pharmaceutical quality standards. There, and perhaps also in other countries, alone in India 77 brands are distributed by 43 companies, aspirin and other pain relieving OTC drugs are exempt from the study of bioavailability [15] with unknown consequences of efficacy and toxicity. In India, the European Medicines Agency (EMA) has recently raised concerns regarding the quality of medicines made in India and distributed across the European Union. Specifically, findings of noncompliance with good clinical practice are currently investigated (EMA Press Release, December 5, 2014).

Summary

Aspirin and salicylic acid are stable compounds and are poorly water soluble at acidic and neutral pH. The solubility is markedly increased in alkaline pH and is also more than 100-fold higher for the respective sodium salts compared to the free acids. Salicylate itself is not applied systemically, but is of key pharmacological interest as the primary metabolite of aspirin *in vivo*.

Salicylic acid has unique physicochemical properties. These are due to the close steric proximity of the acetate hydroxyl group to the carboxyl group. This allows formation of a chelate ring structure and facilitates the release of protons with decreasing pH. The major functional consequence is the accumulation of salicylate inside the cell, specifically in mitochondrial membranes. There the compound acts as protonophore. The consequence is the uncoupling of oxidative phosphorylation because of the abolition of the mitochondrial membrane impermeability for protons. Neither aspirin nor other salicylates exhibit comparable physicochemical properties.

Aspirin is available in hundreds of different medications worldwide. Many of these generics, specifically OTC drugs, may exhibit insufficient pharmaceutical quality standards – with unknown because uncontrolled efficacy and toxicity in comparison to standard aspirin brands. A newly developed micronized aspirin formulation that exhibits markedly improved release kinetics might replace standard plain aspirin as an OTC antipyretic analgesic in the near future.

References

1 Büge, A. (1977) Zur Chemie der Salicylsäure und ihrer wichtigsten Derivate, in *100 Years of the Salicylic Acid as an Antirheumatic Drug* (ed. H Bekemeier), Martin-Luther-Universität Halle-Wittenberg.

2 Kim, Y. and Machida, K. (1986). Vibrational spectra, normal coordinates and infrared intensities of aspirin crystal. *Chem. Pharm. Bull. (Tokyo)*, **34** (8), 3087–3096.

3 Wheatley, P.J. (1964) The crystal and molecular structure of aspirin. *J. Chem. Soc.*, 6036–6048.

4 Bond, A.D., Boese, R., and Desiraju, G.R. (2007) On the polymorphism of aspirin: crystalline aspirin as intergrowths of two "polymorphic" domains. *Angew. Chem., Int. Ed.*, **46** (4), 618–622.

5 Bond, A.D., Boese, R., and Desiraju, G.R. (2007) On the polymorphism of aspirin. *Angew. Chem., Int. Ed.*, **46** (4), 615–617.

6 Sakurai, T. *et al.* (2012) Characterization of the beta-D-glucopyranoside binding site of the human bitter taste receptor hTAS2R16. *J. Biol. Chem.*, **285** (36), 28373–28378.

7 McTavish, J.R. (1987) Aspirin in Germany: the pharmaceutical industry and the pharmaceutical profession. *Pharm. Hist.*, **29** (3), 103–115.

8 Gutknecht, J. (1990) Salicylates and proton transport through lipid bilayer membranes: a model for salicylate-induced uncoupling and swelling in mitochondria. *J. Membr. Biol.*, **115** (3), 253–260.

9 Whitehouse, M.W. (1964) Biochemical properties of anti-inflammatory drugs: III. Uncoupling of oxidative phosphorylation in a connective tissue (cartilage) and liver mitochondria by salicylate analogues: relationship of structure to activity. *Biochem. Pharmacol.*, **13**, 319–336.

10 Horsch, W. (1979) The salicylates] Die salicylate. *Pharmazie*, **34** (9), 585–604.

11 Voelker, M. and Hammer, M. (2012) Dissolution and pharmacokinetics of a novel micronized aspirin formulation. *Inflammopharmacology*, **20** (4), 225–231.

12 Gordon, M.S., Ellis, D.J., and Molony, B. (1994) *In vitro* dissolution versus *in vivo* evaluation of four different aspirin products. *Drug Dev. Ind. Pharm.*, **20**, 1711–1723.

13 Blume, H. and Siewert, M. (1986) Zur Qualitätsbeurteilung von acetylsalizylsäurehaltigen Fertigarzneimitteln: 1. Mitteilung: Vergleichende Reihenuntersuchung zur pharmazeutischen Qualität handelsüblicher ASS-Monopräparate. *Pharm. Ztg. Wiss.*, **47**, 2953–2958.

14 Siewert, M. and Blume, H. (1988) Zur Qualitätsbeurteilung von acetylsalizylsäurehaltigen Fertigarzneimitteln: 2. Mitteilung: Untersuchungen zur Chargenkonformität der biopharmazeutischen Eigenschaften handelsüblicher ASS-Monopräparate. *Pharm. Ztg. Wiss.*, **131**, 21–28.

15 de Oliveira, O.M. and de Melo, E.B. (2013) Quality assessment of samples of generic and similar aspirin tablets (500mg) marketed in Brazil. *Rev. Bras. Farm.*, **94** (1), 35–40.

1.2.2 Determination of Salicylates

Measurement of salicylates is of interest for several purposes. One reason is the control of plasma levels to verify that these are within the therapeutic range. Determination of plasma levels is also necessary in case of intoxication and for controlling the efficacy of detoxification procedures. Plasma or urinary levels of salicylates allow checking for patients' compliance, an important issue for long-term aspirin use in cardiovascular prophylaxis and a frequent natural explanation of so-called aspirin "resistance" (Section 4.1.6). Finally, measurements of salicylates and their metabolites are of interest to study the pharmacokinetics of the compounds, in particular in studies on drug metabolism and drug interactions. Measurement of salicylate as a surrogate parameter for its "precursor" aspirin is easier and sufficient in some cases, for example, salicylate intoxication (Section 3.1.1).

The therapeutic plasma levels of salicylate are dose dependent and vary in dependency on the indication. They are in the range of 10 µg/ml in antiplatelet doses, 50–100 µg/ml in analgesic and 100–200 µg/ml in anti-inflammatory doses. The plasma levels of unmetabolized acetylsalicylic acid are considerably lower and largely dependent on the formulation: The more rapid the absorption, the higher the peak level of unmetabolized acetylsalicylic acid.

1.2.2.1 Gas–Liquid Chromatography

Gas–liquid chromatography (GLC) is the reference standard. The technique allows separate determination of acetylsalicylic acid, salicylic acid, and its metabolites. The detection limit is about 1 µg/ml.

1.2.2.2 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is an alternative to GLC, but more complex and time-consuming. Reversed-phase HPLC techniques with photometric detection are the methods of choice [1]. These have the advantage that the complete spectrum of aspirin and its metabolites can be measured simultaneously. However, one problem associated with this type of assay is the spontaneous hydrolysis of aspirin to salicylate in protic solvents, including water and methanol but also plasma (Section 2.1.1). Thus, some degradation of aspirin may occur *ex vivo* during sample processing. A modification of this technique for human plasma, including extraction of salicylates in organic solvents, allows simultaneous determination of aspirin and its metabolites down to levels of 100 ng/ml with an interassay variation of less than 10% (Figure 1.2.2-1). This technique combines simplicity in sample treatment with stability of aspirin over several days (!) without significant decomposition [2].

More recently, another sensitive ultrahigh-performance liquid chromatography has been described, again using methylbenzoic acid as internal standard. The detection of salicylate and unmetabolized acetylsalicylic acid was linear between 0.2 and 200 µg/ml (correlation coefficient >0.999) with a detection limit of 0.17 µg/ml for both compounds [3]. With one exception (tilcitin), the assay was insensitive to more than 60 drugs, possibly coadministered to cardiac patients – however, other antiplatelet drugs were not included. This method has been successfully used to determine aspirin and salicylate plasma levels in healthy volunteers and patients with coronary artery disease treated with low-dose aspirin (Table 1.2.2-1).

1.2.2.3 Spectrophotometry

Spectrophotometry is the earliest and most widely used method for measuring serum salicylate levels. The classical assays are colorimetric assays, taking advantage of the intense red color of salicylate/Fe^{3+} complexes (Gerhardt reaction). The technology is simple and particularly suitable for compliance measurements.

Trinder Method The Trinder method [4] is a colorimetric test where salicylic acid is determined by measuring the absorbance of the ferric ion–salicylate complex after total serum protein is precipitated by mercuric chloride and allowed to react

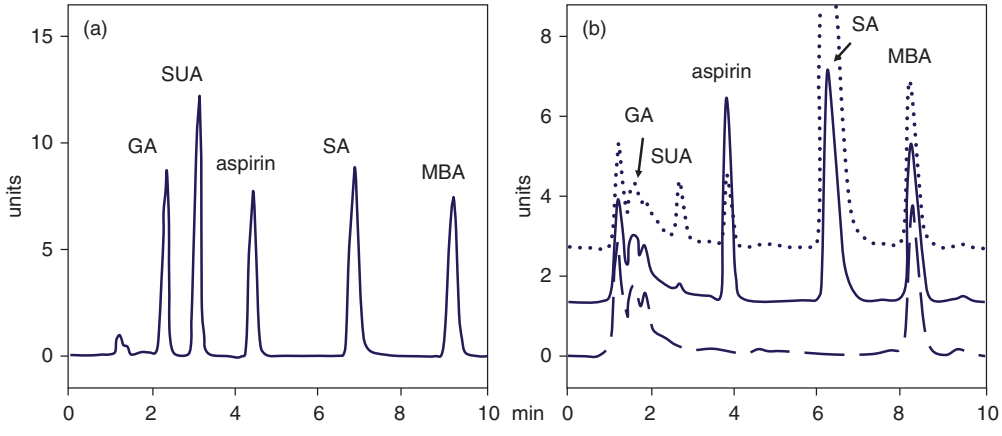


Figure 1.2.2-1 Chromatograms of a standard mixture of acetylsalicylic acid and major metabolites (50 ng each) (a) and plasma levels of a volunteer before, 10 min after, and 1 h after oral administration of 500 mg aspirin (b). (Modified after Ref. [2]).

with ferric iron supplied by ferric nitrate. This results in the generation of a complex of salicylate and ferric ion. The maximum absorption (A_{max}) of the ferric complex is 540 nm. Quantification is done by measuring light absorbance at this wavelength in a spectrophotometer.

The Trinder method is simple, rapid, and inexpensive. This method has been used almost exclusively in the past, when a detection limit of about 100 µg/ml of salicylate was sufficient. The Trinder method solely measures salicylates, *not* acetylsalicylic acid. (False) Positive results may be

Table 1.2.2-1 Plasma levels of acetylsalicylic acid (ASA) and salicylic acid (SA) in healthy volunteers and patients with stable coronary artery disease (CAD) before and 1, 6, and 24 h after intake of one 75 mg non enteric-coated aspirin tablet.

Group	ASA				SA			
	Before	After			Before	After		
		1 h	6 h	24 h		1 h	6 h	24 h
Healthy								
Volunteers mean	<0.2	0.65	<0.2	<0.2	<0.2	4.34	0.75	<0.2
($n = 10$) SD	n/a	0.26	n/a	n/a	n/a	1.35	0.32	n/a
CAD								
Patients mean	n/a	0.52	n/a	<0.2	n/a	3.77	n/a	<0.2
	n/a	0.27	n/a	n/a	n/a	1.48	n/a	n/a

No differences between volunteers and patients (on a number of additional drugs, see text) were observed.
Data are µg/ml as mean ± SD. n/a: not analyzed [3].

obtained with salicylamide or methyl salicylate. Conversely, the method can also be used to measure these compounds, for example, in case of poisoning. However, the Trinder assay is rather nonspecific and sensitive to a large number of other acids and amines [5]. This also includes compounds and their metabolites that might be increased in patients with Reye syndrome where the Trinder assay frequently was used for determination of salicylate levels. Interestingly, salicylate levels in liquor and serum of children with Reye's syndrome measured with sensitive and selective HPLC methods were reportedly only 1% of those measured by the Trinder assay [6] (Section 3.3.3.2).

Second-Derivative Synchronous Fluorescence Spectrometry (SDFSFS) Another method that allows for simultaneous determination of acetylsalicylic acid and its major metabolites in one assay is second-derivative synchronous fluorescence spectrometry (SDFSFS) [7]. This method appears to be the first nonchromatographic technology for the simultaneous determination of aspirin and its major metabolites in one single serum sample. The technique is not sensitive to several other drugs, found frequently in the sera of patients suffering from inflammatory diseases (antipyrine, ibuprofen, indomethacin, theophylline, and others).

Summary

Several reliable methods are available to determine aspirin and its major metabolites in biological fluids, including plasma (serum), liquor, synovial fluid, and urine. Most of them have the necessary sensitivity (detection limit 0.5 μg/ml or less).

HPLC separation and subsequent identification of the spots by appropriate standards is the most frequently used technology. Advantages are the simplicity and reproducibility of the method, high sensitivity, and the possibility for simultaneous determination of several aspirin metabolites together with aspirin itself in one and the same sample. Disadvantages of this and some other technologies are the spontaneous (pH-dependent) and enzymatic hydrolysis of aspirin. However, this problem can be solved by appropriate sample processing.

The Trinder method, a colorimetric assay, was historically the most frequently used method for salicylate determination. This assay exhibits a number of cross-reactions with other chemicals that might become particularly relevant in hepatic failure and possibly has caused false-positive results in Reye's syndrome. GC/MS is clearly the most reliable technology. However, it needs expensive equipment and experienced investigators.

References

1 Klimes, J. *et al.* (1992) Simultaneous high-performance liquid chromatographic determination of salicylates in whole blood, plasma and isolated erythrocytes. *J. Chromatogr.*, **584** (2), 221–228.

2 Kees, F., Jehnich, D., and Grobecker, H. (1996) Simultaneous determination of acetylsalicylic acid and salicylic acid in human plasma by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Appl.*, **677** (1), 172–177.

3 Rubak, P. *et al.* (2013) Low-dose acetylsalicylic acid therapy monitored with ultra high performance liquid chromatography. *Clin. Biochem.*, **46** (12), 988–992.

4 Trinder, P. (1954) Rapid determination of salicylate in biological fluids. *Biochem. J.*, **57** (2), 301–303.

5 Kang, E.S. *et al.* (1983) Measurement of true salicylate concentrations in serum from patients with Reye's syndrome. *Clin. Chem.*, **29** (6), 1012–1014.

6 Andresen, B.D. *et al.* (1982) Aspirin and Reye's disease: a reinterpretation. *Lancet*, **1** (8277), 903.

7 Konstantianos, D.G. and Ioannou, P.C. (1992) Simultaneous determination of acetylsalicylic acid and its major metabolites in human serum by second-derivative synchronous fluorescence spectrometry. *Analyst*, **117** (5), 877–882.