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Chapter

Salicylic Acid Sans Aspirin in Animals and Man

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Abstract

Analyses in non-aspirin takers finding salicylic acid (SA) and hydroxylated metabolites in serum also SA and salicyluric acid (SU) in urine led to a re-evaluation of dietary sources of salicylates. Fruit and vegetable sources explained higher levels found in drug-free vegetarians, which overlapped with those from patients on low dose aspirin. That drug's chemo-protective action in cancer is, at least partially, attributable to its principal metabolite, SA—which we believe contributes to the benefits of a vegetarian diet. However, diet is unlikely to be the sole source of the circulating salicylate found in aspirin-free animals and man. We adduced evidence for its persistence in prolonged fasting and biosynthesis in vivo from labelled benzoic acid. We review the roles, defined and potential, of SA in the biosphere. Emphasis on the antiplatelet effect of aspirin in man has detracted from the likely pivotal role of SA in many potential areas of bioregulation—probably as important in animals as in plants. In this expanding field, some aspirin effects, mediated by apparently conserved receptors responding to SA, are discussed. The perspectives revealed may lead to re-evaluation of the place of salicylates in therapeutics and potentially improve formulations and drug delivery systems.

Keywords: salicylic acid, salicylic acid metabolism, dietary sources, biosynthesis, homologue receptors, conserved effects, siderophores, aspirin

1. Introduction

Salicylic acid (SA) in plants is a ubiquitous compound shown to be pivotal in initiating the response to a variety of physical, chemical and biological insults [1]. Analgesic and antipyretic properties of plant extracts, notably from willow, meadowsweet and myrtle, had been known for many centuries before isolation of SA as the active principle. Since synthesis of its acetyl ester—aspirin—investigation has focused on the properties of that compound which is rapidly hydrolysed (serum $t_{1/2}$ of 20 min) to SA which itself has a half-life of 2–4 h [2]. The demonstration that aspirin works by serine side chain acetylation of Cox-1 and Cox-2 isoforms has detracted attention from SA itself; that compound, despite weak reversible Cox 1 and absent Cox 2 inhibition, is as effective as aspirin in vivo in suppressing inflammation [3]. Inhibition of transcription of the Cox-2 gene by micromolar concentrations of SA is the likely explanation [4].

2. SA without aspirin (SA sans ASA)

Investigating the possible use of low dose aspirin as an aromatic probe to measure hydroxyl free radicals by assessing the hydroxylation of SA to form 2,3 and 2,5

dihydroxybenzoic acids (DHBAs)—**Figure 1**—required a sensitive HPLC assay with appropriate controls. That work revealed the presence of substances which had identical retention times to SA, 2,3 DHBA and 2,5 DHBA in the serum extracts of subjects not taking aspirin. The exclusion of contamination was followed by studies to determine the authenticity of these substances as SA, 2,3 DHBA and 2,5 DHBA.

2.1 SA in blood

Examination of the chromatograms of blank serum or plasma from published methods of SA analysis revealed the presence of an unknown substance with a retention time (R_t) similar to SA [5, 6]. While Ruffin et al. [7] reported SA in the plasma from 17 of 53 subjects at baseline there was no information as to how they confirmed identification of the compound.

We examined samples from drug free volunteers: extracts of acidified serum were analysed by high performance liquid chromatography (HPLC) with electrochemical detection. Chromatographic conditions were altered and the R_ts of the unknown compounds compared against authentic SA, 2,3 DHBA and 2,5 DHBA. Serum samples (some spiked with SA) were also incubated with a bacterial salicylate hydroxylase and the substance which had a R_t identical to SA disappeared. Finally the trimethylsilyl (TMS) derivative of the unknown and SA had, using gas chromatography–mass spectrometry (GC–MS), a similar retention time and total ion chromatogram [8].

2.2 SA and salicyluric acid (SU) in urine

Armstrong et al. [9] had detected, by paper chromatography, a compound with characteristics similar to those of SU in the urine of 400 people who had not taken salicylate drugs. That was in an admixture of 49 compounds of predominantly, it was suggested, dietary origin. Von Studnitz and colleagues, who also used a paper system, suggested SA might be one of the phenolic acids in the urine of subjects *on a*

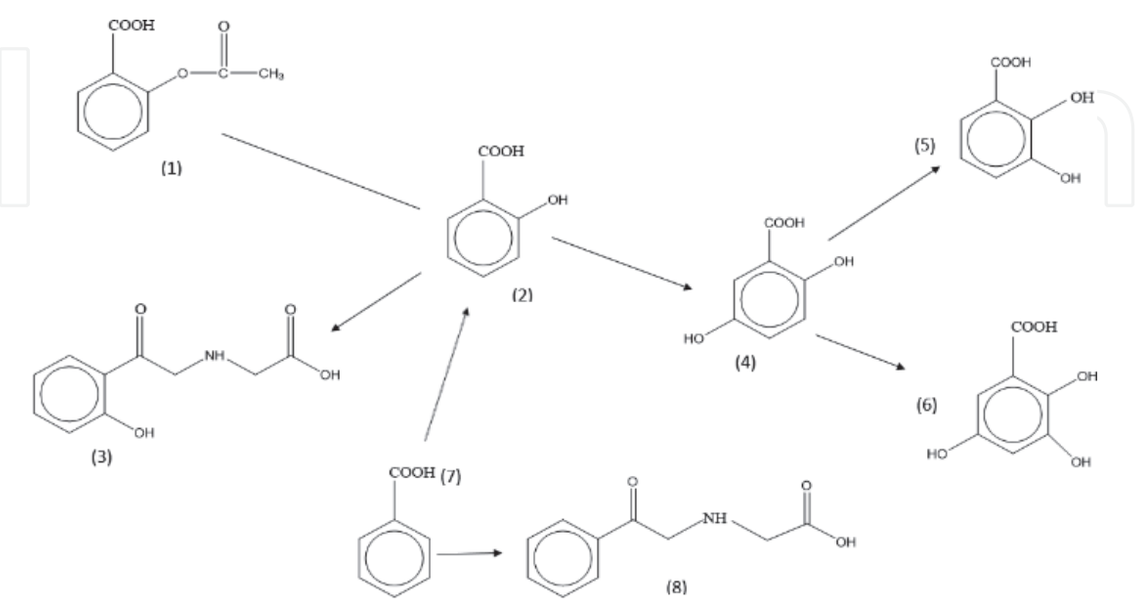

Figure 1.
Metabolism of aspirin, salicylic acid and benzoic acid. (1) acetylsalicylic acid (aspirin); (2) salicylic acid; (3) salicyluric acid; (4) 2,5 dihydroxybenzoic acid; (5) 2,3 dihydroxybenzoic acid; (6) 2,3,5 trihydroxybenzoic acid; (7) benzoic acid; and (8) hippuric acid.

diet restricted to glucose and citric acid [10]. Young reported a compound with thin layer chromatographic properties of SU [11] in a single individual on a synthetic diet, while Finnie and co-workers found a similar compound on their paper system in children not taking salicylate drugs [12]. At the time of all these earlier investigations methods for adequately characterising and quantifying the purported SA and SU were not readily available.

Other work [13] designed to assess the dietary importance of salicylates had examined acid-treated urine using HPLC with fluorescence detection but salicylates other than SA, salicylate precursors and structurally related compounds may have been included in the values reported.

We examined 24 h urine samples from 10 volunteers who had not taken any salicylate drugs during the previous 2 weeks. The acid hydrophobic compounds (=organic acids) were separated using HPLC and quantified electrochemically. The R_ts of the extracted substances and those of SA and SU were compared under two sets of chromatographic conditions and found very similar to those of authentic substances. The unknown substances, isolated by HPLC, and treated with acetyl chloride in methanol were compared with the methyl esters of SA and SU using GC–MS. After esterification the unknown compounds had mass spectra and R_ts comparable to those of methyl-SA and methyl-SU [14].

These findings in serum and urine led us on to explore, in some detail, the likely dietary sources for the salicylate compounds we found and confirmed. At the time interest was re-awakening in the potential benefits of salicylates commonly found in the diet [13]—as opposed to what were considered traditional plant medicine sources.

3. Dietary source of salicylates

3.1 Previously

The occurrence of "natural salicylates", such as SA, in strawberries and other fruits was raised in the Lancet in 1903 [15] and the matter of whether these natural salicylates were superior to synthetic salicylates was the subject of a JAMA editorial in 1913 [16]—no superiority was concluded! Interest then appeared to wane until re-invigorated by the popularity, from ~1970, in therapeutic trials—arising from the apparent cross-reactivity of tartrazine and aspirin—of exclusion diets.

3.2 Background

Many plant derived non-nutritive compounds exert, in mammalian systems, biological activities that may have an impact on health and disease risk [17] and we proposed [18] that SA might provide a link between aspirin, diet and the prevention of colorectal cancer (CRC). There is good evidence that the regular intake of aspirin decreases the risk of developing cancer [19]. A mechanism for platelet mediated CRC tumorigenesis has been proposed [20]; that would, of course not be attributable to SA itself but a more balanced view is that both constituent groups of aspirin (acetyl and SA moieties) contribute to the anti-cancer effects [21].

Assessment of the extent of the contribution of diet to SA in blood and urine cannot be easily inferred from direct analysis of its concentration therein. There is considerable variability in peak serum levels of SA in subjects receiving a standard dose [7] while urinary salicylate is influenced by urine flow, pH, the presence of other organic acids and the saturability of SU formation and/or excretion [2].

3.3 Salicylates from food

It was unclear whether sufficient salicylic acid could be obtained from dietary sources to influence health and disease with estimated daily intakes ranging from 0.4 to 200 mg/day [13, 22, 23]. That range reflected the disparate information available on the salicylate content of foods.

Comparison, in the serum of subjects not taking aspirin, of SA levels in 37 vegetarians and 39 non-vegetarians found higher concentrations in the former [24]. That study revealed median concentrations of 0.11 (range 0.04–2.47) μmol/L and 0.07 (range 0.02–0.20) μmol/L respectively: the median of the difference was 0.05 μmol/L (95% confidence interval for difference 0.03–0.08; $p < 0.0001$). The median SA level measured in serum from 14 patients on aspirin 75 mg/day was, at 10.03 (range 0.23–25.40) μmol/L, significantly higher. However there was overlap in serum SA concentrations between the vegetarians (8 higher than lowest low dose aspirin) and patients taking aspirin (6 below the highest vegetarian value). These findings should be considered in light of the inhibition of COX2 transcription that has been shown to occur at SA levels as low as 0.1 μmol/L [4].

In a further study the urinary excretion of SA and SU was assessed in 24 h samples from 27 non-vegetarians, 21 vegetarians and 40 patients taking 75 or 150 mg aspirin/day [25]. For SU, the principle urinary salicylate, vegetarians excreted significantly more than the non-vegetarians (median 11.01; range 4.98–26.60 μmol/24 h compared with 3.91; range 0.87–12.23 μmol/24 h) but these amounts were significantly lower than those excreted by patients on aspirin. Significantly more SA was excreted by the vegetarians (median 1.19; range 0.02–3.55 μmol/24 h) than by the non-vegetarians (median 0.31; range 0.01–2.01 μmol/24 h). The median amounts of SA excreted by the vegetarians and the patients taking aspirin were not significantly different. These values were comparable to those found earlier, using less specific methodology, in a group of drug free volunteers on a variety of diets [13].

3.3.1 The spice of life

Awareness that certain spices had been reported [22] to contain especially high concentrations of SA and the reported very low incidence of colorectal cancer in rural India [26] led to our particular assessment of spices.

Spices, Indian cooked dishes and blood and urine samples taken after ingestion of a test meal were investigated for their salicylate content. Total salicylate content determination required a preliminary alkali treatment step before our standard extraction [27] as, in plants, phenolic glycosides and carboxylic esters are present as well as the “free” phenolic acid. Our standard assay conditions for SA were then applied. All samples of spices and cooked meals examined contained SA (up to 1.5 wt%); cumin, turmeric, red chilli powder, paprika and cinnamon were especially rich sources. Our measurements were considerably higher than those previously published [22]. That was attributed to previously suboptimal extraction, chromatographic separation and detection [27]. The identity of the SA fractions (on HPLC) from cumin, paprika and turmeric was confirmed, after elution and esterification, by GC–MS of their methyl esters.

The potential bioavailability of SA derived from a prepared meal was assessed in a single aspirin free volunteer after a 10 h fast. Consumption of 545.3 g of a cooked vegetable dish (shown by aliquot assay to contain 94.03 g of total salicylates) was followed by regular blood and urine collection over 6 h. Serum SA doubled within 1.5 h and urinary SU increased ~20-fold during that time.

Native Indian volunteers, living in a rural area near Chennai, had been recruited for another study as representative of that community for health lifestyle and nutritional status. They had a diet of locally grown vegetables, grains and pulses flavoured with spices and herbs. The serum from these 21 South Indians had a median SA concentration of 0.263 μmol/L (range 0.05–0.64—significantly higher (~2.5- to 3.5-fold higher) than those found in the sera of the other groups reported above [24]; $p < 0.001$ against both vegetarians and non-vegetarians by Mann–Whitney *U* test) [27]. Summarised and compared with other results below in **Table 1**.

	Median (μmol/L)	Range (μmol/L)
Vegetarians n = 37	0.110	0.04–2.47
Non-vegetarians n = 39	0.070	0.02–2.00
Southern Indian villagers n = 21	0.263	0.05–0.64
75 mg aspirin takers n = 14	10.03	0.23–25.4

Limit of detection of the method = 0.005 μmol/L.
***Tables 1** and 2—with modification—from Ref. [28]—https://pubs.acs.org/doi/abs/10.1021/jf800974z?src=recsys: further permissions regarding use of the content should be directed to the ACS.*

Table 1.
Results in Man [24, 27].

3.4 SA in food

Given that SA is a stress hormone in plants we can anticipate that locality, varietal and growing conditions could affect total salicylate content at harvesting before any variability in processing conditions and storage effects. Wide reported ranges for different brands assayed using standard conditions are therefore not particularly surprising. For example the SA content of five brands of orange juice obtained from Scottish retailers ranged from 0.47 to 3.01 mg/kg [29].

3.4.1 Organic or not?

Usually an open question but, given the above considerations, probably not in respect of SA content. Thus the median SA contents in organic and non-organic vegetable soups were 117 ng/g (range 8–1040) and 20 ng/g (range 0–248) respectively; the median between the difference groups was 59 ng/g (95% confidence intervals 18–117 ng/g), $p = 0.032$ by Mann Whitney *U* test [30]. Consider also constraints of drought, other physical stresses and non-availability of pest control which inevitably prevail in many emergent nations.

3.4.2 A Scottish overview

Clearly sample selection and methodology will affect estimates of SA content in the diet. Using the assay methodology our group developed [27, 30] to supplement published results, Wood et al. [29] prepared a comprehensive dietary database. They filtered published results of dietary constituent total salicylate content by specific criteria. Food items had to be randomly selected and purchased from various commercial outlets at different times of the year; food samples to be prepared using standard domestic practices; optimised sample extraction and hydrolysis conditions were to be clearly described or cited, and salicylate determination to be based on modern techniques of HPLC and mass spectrometry with validation

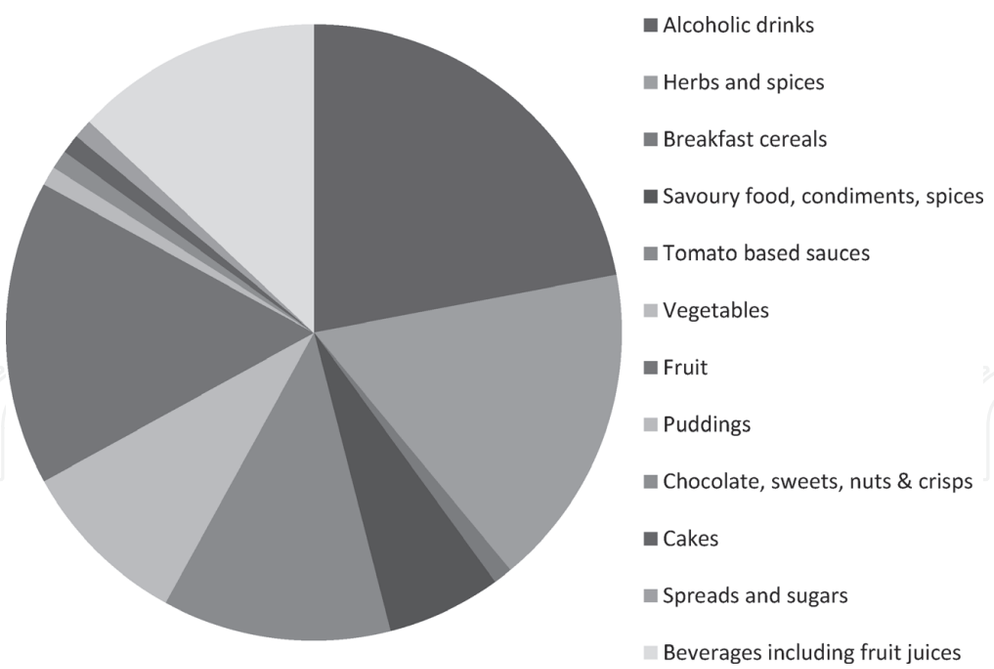

Figure 2.
Relative contributions of different food groups to total salicylate intake of a Scottish population. Source: [29].

and quality assurance methods summarised. Combination of such published data, which met these criteria, together with their in-house analyses resulted in a database of 27 types of fruit, 21 vegetables, 28 herbs, spices and condiments, 2 soups and 11 beverages—expressed as median values to reflect the non-normal distribution of the results.

Subsequently dietary intake was assessed by applying this salicylate database, using a validated questionnaire, to 237 healthy individuals age range (17–72) from Aberdeen [29]. Estimated median total salicylate intakes for men and women were 4.42 and 3.16 mg/day respectively, a gender difference not sustained when corrected for energy. Primary food sources of salicylates as shown in **Figure 2** were alcoholic beverages (22%), herbs and spices (17%), fruits (16%), non-alcoholic beverages including fruit juice (13%), tomato-based sauces (12%) and vegetables (9%). Salicylate intake was significantly ($p < 0.001$) and positively associated with intakes of fibre, potassium, vitamin C and alcohol!

4. Endogenous salicylic acid

4.1 Background

There were clear suggestions in the literature that SA and SU in urine might not all derive from diet, given their presence in the urine of subjects on restricted diets [9, 11]. In addition quantification of the contribution from fruit and vegetable consumption to circulating SA levels in man had demonstrated, using a sensitive and specific method that <20% of the variability derived from these sources [31]. So some circulating SA in aspirin-naïve or -free individuals may derive from other dietary sources or be of non-dietetic origin.

4.2 SA in animal blood

Blood samples, serum or plasma, were obtained from animals at London Zoo or the Department of Biological Services, University of Glasgow in accordance with

Animal	Phylogenetic class	SA (μmol/L)
Burrowing Owl	Aves	9.854
Ne-Ne	Aves	5.609
Indian Rhinoceros	Mammalia	4.700
Pigmy Hippopotamus	Mammalia	2.384
Agouti	Mammalia	2.116
Asian Elephant	Mammalia	1.635
Burmese Python	Reptilia	1.362
Rabbit	Mammalia	1.129
Piglet	Mammalia	1.010
Arabian Onyx	Mammalia	0.777
Sheep	Mammalia	0.715
Tiger[a]	Mammalia	0.661
Brown Trout	Pisces	0.538
Giraffe	Mammalia	0.507
Donkey	Mammalia	0.473
Sacred Ibis	Aves	0.353
Goat	Mammalia	0.310
Giant Anteater	Mammalia	0.293
Collared Peccary	Reptilia	0.237
African Lion[a]	Mammalia	0.226
Cow	Mammalia	0.216
Gelada Baboon	Mammalia	0.210
Chinese Alligator	Mammalia	0.156
Domestic Cat	Mammalia	0.144
Pond Heron	Aves	0.136
Gorilla	Mammalia	0.125
Monkey*	Mammalia	0.080
Mouse	Mammalia	0.078
Rat	Mammalia	0.069
Domestic Cat**	Mammalia	0.058
Chimpanzee	Mammalia	0.033
Crab***	Crustacea	<0.005
Prawn	Crustacea	<0.005

[a]*Mean concentration in five animals.*
**Red faced spider monkey.*
***Fed only meat.*
****European shore crab.*

Table 2.
Concentration of salicylic acid (SA) in the blood or body fluid of a variety of animals.

their approved codes of practice. The results (**Table 2**) showed that species regarded as primarily carnivorous had blood SA levels comparable to those measured in herbivores [28]. The highest levels detected were in the range associated

with aspirin use in man and only the crustacean body fluid samples examined did not contain SA.

As some bacteria, notably *Mycobacterial*, *Yersinia* and *Pseudomonas* species, synthesise SA to enhance iron chelation (see Section 5.1) the possibility of a gastrointestinal particularly colonic, bacterial source for SA was assessed in two animal models. Pooled serum from six mice treated with neomycin 100 mg/kg/day for 4 days had a serum SA concentration which was, at 0.309 μmol/L, slightly higher than the level of 0.268 μmol/L measured in six untreated animals. Other measurements were done on serum samples from Sprague-Dawley rats delivered by caesarean section, raised in a sterile environment and fed sterilised food. A group of 8 such germ free animals had a pooled serum SA level which was, at 0.166 μmol/L, ~2.5× greater than the level of 0.069 μmol/L in serum from a group of control animals [28].

4.3 SA in diet-restricted and fasting human subjects

A preliminary study followed serum SA and urinary SA + SU over 3 days on a water/milk diet (confirmed salicylate free on analysis) in a subject free of aspirin for at least 2 weeks. Excretion of SA + SU continued at a rate of ~2.1 μmol/24 h throughout and serum SA did not fall—over the 72 h of the study—below 0.1 μmol/L (20× the limit of detection of the assay) [28].

In six patients who had total colectomy or rectal excision following standard preoperative bowel preparation low level serum SA (range 0.012–0.085 μmol/L) was detected and urinary SA + SU excretion persisted (median lowest level 0.613 μmol/24 h; range 0.184–7.607) in all subjects for up to 5 days postoperatively, rising only on refeeding [28]. These results also, of course, have some relevance to Section 4.2.

4.4 SA formation from benzoic acid

Benzoic acid (BA) is a natural constituent of plants, with high levels found in fruits and vegetables. In plants synthesis of SA derives—at least partially—from phenylalanine via cinnamic and benzoic acids. Prior work, using formula diet feeding, also demonstrated that hippuric acid, the main metabolite of BA, may be formed endogenously in man, while a Sprague-Dawley rat radiolabeled experiment showed phenylalanine as the likely precursor [32]. Sodium benzoate as a food preservative also contributes to human intake and very high doses have been used in hepatic encephalopathy. These considerations led us to determine whether addition of BA to a very carefully standardised diet produced any change in serum or urinary salicylates—see **Figure 1**.

A preliminary study, over 4 days in two subjects, suggested that BA, 1 or 2 g/day on days 3 and 4 might be associated with a modest increase in urinary SA + SU excretion. Subsequently a labelled study was undertaken over 3 days in six individuals (4 M, 2F) who received 1 g of uniformly ring-labelled ^{13}C BA with each of their main meals on day 2. They replicated their carefully recorded day 1 diet throughout and had regular blood sampling with complete urine collections. The **total** SA + SU urinary excretion increased, but not significantly ($p = 0.052$) and only in the 8–16 h sample after the first dose of BA. While no ^{13}C was detected in samples prior to ingestion of the BA, the ^{13}C isotope was confirmed in the 8–16 h urine sample from all six subjects. Its presence was determined by preliminary GC fractionation before subjecting the relevant fractions to derivatization and GC–MS. The ^{13}C isotope accounted, by selective ion monitoring, for 0.4–10.9% (median 3.4%) in the SA derivative and 6.8–43.1% (median 33.9%) in the SU derivative. In addition considerable amounts of the expected $^{13}C_6$-labelled hippuric acid were found [28].

Set against the **total** SA + SU levels the extent of the SU $^{13}C_6$ labelling found might be considered surprising but could, as well as confirming the in vivo synthesis of SA, point to possible bioregulation of the levels of endogenous serum SA.

5. Salicylic acid in the biosphere

The protean actions of aspirin in animals and man are here, in a short review by JRL an GJB, set against what is known of the role of its SA precursor in earlier life forms.

5.1 Bacteria

This complex area is here only briefly overviewed in relation to its potential for pointing to possible effects of SA preserved into animals.

Para-aminosalicylic acid (PAS), the earliest truly effective anti-tuberculous agent, was long thought an analogue for para-aminobenzoic acid and so an inhibitor of folic acid biosynthesis. That was before the discovery of the mycobacterial siderophore (iron binding molecule) mycobactin, and that SA (also formed, as an extracellular metabolite, by mycobacteria in iron deficient conditions) is its direct precursor. It appears PAS primarily inhibits the conversion of SA into myobactin. Possible secondary roles for SA are the transfer of Fe^{2+} across the cell membrane, either for direct incorporation into various porphyrins and apoproteins, or for storage of iron within the cytoplasm in bacterioferritin (both roles also potential targets for PAS) [33].

There are many kinds of bacterial siderophores but SA or one of its hydroxylated metabolites (2,3DHBA) are at the core of the aryl- capped molecules found in *E. coli* (Enterobactin); *Yersinia* sp. and *Klebsiella pneumoniae* (Yersinibactin); *Pseudomonas* sp.(Pyochelin); *Vibrio* sp. (Vibriobactin/Vulnibactin) and *Acinetobacter baumanii* (Acinetobactin) [34]. These authors described a probe for the initial aryl acid activation enzymatic step in the synthetic pathways of these "bactins" (*via a non-ribosomal peptide synthetase pathway initiated by adenylation*) and suggested lack of human homologues makes this a potential drug target—but see Section 5.3.4.

Intriguingly investigation into the bioinorganic chemistry of bacterial siderophores has revealed that many have functional capacities other than mere iron homeostasis. Examples include interactions with other metals such as zinc, copper and boron; signalling agents (referred to as "ferrimones") in the regulation of genes related to iron metabolism; protection—by those with catecholate structures—from oxidative stress and an antibiotic function in sideromycins [35].

Finally bacterial growth in the presence of salicylate can be both beneficial and detrimental. On the one hand an intrinsic multiple antibiotic resistance phenotype can be induced and on the other reduced resistance to some antibiotics might result and bacterial virulence factors may be affected [36]. While the in vivo consequences of these observations is speculative the findings highlight, the authors suggest, the ability of salicylate to alter gene expression; they claim that the only life form not yet (then) shown to be affected by salicylate is the Archaea!

5.2 Plants

While salicylic acid (initially from plant sources) has been used in therapeutics for millennia detailed knowledge of its role in plants is relatively recent. Although plant phenolics are diverse and ubiquitous they were traditionally assumed to be unimportant secondary metabolites but SA in plants is a critical hormone playing a direct role in the regulation of many aspects of growth and development as well as

in thermogenesis and disease resistance [37]. The first clear evidence came, intriguingly (in relation to its antipyretic qualities in animals), from its role in voodoo lily thermogenesis; that appears to be mediated by stimulation of the mitochondrial alternative respiratory pathway [38]. Soon thereafter its role as a defence signalling hormone was documented—though the ability of plants to develop acquired immunity after pathogen infection was first proposed many years earlier. In the acquired immunity—called "systemic acquired resistance" (SAR)—of plants to biotrophic (i.e. threatening living cells) pathogens, the role of SA is pivotal. Careful study has identified two pathways for its synthesis, numerous proteins that regulate its synthesis and metabolism and some signalling components, including a large number of potential targets/receptors, which operate downstream of SA [39]. This is a perplexing field; for example while the non-specialist can readily appreciate methylation of SA to a volatile ester for transport through the phloem (before demethylation at a site where SA levels are low) subsequent steps are complex. As these authors point out it is increasingly evident that SA does not signal immune response by itself but as part of an intricate network of other plant hormones. We would highlight, from the viewpoint of the present review, their suggestion that it is important to differentiate SA "targets" from the subset (whose criteria, they concede, will be difficult to specify) that meet additional conditions to be designated "receptors" [40]. That idea is particularly relevant when later considering the propensity of aspirin to acetylate many animal protein "receptors"—see Section 5.3.4.

The wide range of basal SA levels between and within plant species, and potential for a biphasic/concentration dependent response may explain some conflicting reports on the spectrum of plant processes it influences. Despite these caveats the long list affected by exogenous SA includes resistance to biotic (pathogen-associated) stress and tolerance to many abiotic stresses (drought, chilling, heat, metal, UV radiation, and salinity/osmotic stress) as well as multiple aspects of plant growth and development. These include photosynthesis, senescence, thermogenesis, respiration, glycolysis, the Krebs cycle and the alternative respiratory pathway [40].

5.3 Salicylic acid and aspirin (ASA) in animals and man

Although the use of willow extracts had been known for centuries the report of its first well documented use—as a cheaper remedy for "the agues" than expensive cinchona bark—focused on its antipyretic properties. Then, particularly in the decades following the isolation of SA as the active principle, evidence steadily accrued for its efficacy as an analgesic and anti-inflammatory in e.g. acute rheumatic fever.

5.3.1 After the discovery of aspirin

Following Hoffman's synthesis of the apparently better tolerated ester in 1897 use of that compound prevailed. ASA was the prototype non-steroidal anti-inflammatory drug (NSAID); it seems that term arose from a need to distinguish it from the undesirable effects of synthetic steroids. As a prodrug for a long recognised active agent its mode of action was naturally linked to the effects of SA.

It was not until the 1960s that work by Vane and Piper led to the proposal of a single mode of action of ASA in the inhibition of prostaglandin synthesis. The resulting paradigm-shifting series of experiments led to the discovery that inhibition of constitutive COX-1 and of COX-2 (predominantly inducible) by serine side-chain acetylation altered levels of prostaglandins and leukotrienes. This revelation came at a time when the potency of ASA as an inhibitor of platelet aggregation in the treatment of vascular disease was coming to the fore. So Vane's work explained,

in a unitary and coherent way, the multiple pharmacological actions of ASA. The ester prevailed—particularly as SA itself had no significant anti-COX-1 effect on platelets.

5.3.2 Platelet effects predominant?

This emphasis arose from the apparent efficacy, in cancer chemo-protection, of ASA at the low doses (~70–100 mg/day) used to inhibit platelet aggregation. Irreversible inhibition of COX-1 in the circulating anucleate platelets ensures that thromboxane A_2 formation is prevented throughout their lifespan without, at these doses, suppressing the production of prostacyclin (PGI_2) which mediates platelet inhibition and vasodilatation. While that is the principle effect required in vascular disease platelet activation also triggers a host of processes leading to leucocyte recruitment into various tissues and subsequent phenotypic changes in stromal cells contributing to atherosclerosis, intestinal inflammation and cancer as well as atherothrombosis [41]. That review also encompasses the non-COX effects of the widespread acetylation of other proteins by ASA—quoting one study which revealed over 12,000 ASA-mediated acetylations in over 3700 proteins!

5.3.3 Do earlier accepted effects of ASA and SA still hold?

There is a trend to describe non-COX, indeed increasingly non-platelet, effects of ASA as "non-canonical". That tag appears to include almost all actions not demonstrably due to COX acetylation with the possible exception of inhibition of COX-2 gene transcription [4].

We should, however, remind ourselves that

a. It is generally accepted that although SA is a much weaker inhibitor of COX activity in vitro their anti-inflammatory effects in vivo are comparable [3].

b. ASA has a very short serum half-life compared with SA [2]; its passage (almost certainly total salicylates were determined) through the blood/brain barrier is slow and incomplete [42]. That observation is particularly relevant to the oft forgotten central action of salicylates [43].

c. ASA's antipyretic effect was first validated centuries ago using plant extracts; it is mainly due to inhibition of COX-2 in the hypothalamus [44].

d. There is a clear dose/response relationship between the analgesic effect of ASA up to a dose of 1.2 g [45] compared with the plateau above ~100 mg/day for the effect on platelets and its efficacy in chemoprevention of colorectal adenomas [46].

5.3.4 ASA "receptors"

The eminent facility for ASA to acetylate proteins has been known for decades and proteomic studies—*see above*—have shown its very marked extent. While the functional relationship between such activity and its effects are unclear the blockade of glucose6phosphate dehydrogenase (G6PD), affecting the pentose phosphate pathway, and disruption of mitochondrial respiration may explain platelet autophagy [41]. Clearly, as for SA in plants, caution is required in the strict definition of ASA receptors [39, 40].

5.3.5 SA "receptors"

While the above caveat applies the blunderbuss masking effect of acetylation is not a consideration. At least 15 SA binding proteins are described to date [39] but some intriguing examples point to effects on proteins with plant and bacterial homologues.

Human glyceraldehyde3-phosphate dehydrogenase (HsGADPH)—has been identified as a SA binding protein—as it is in plants. In addition to its central role in glycolysis GADPH participates in pathological processes, with effects on viral replication and neuronal cell death [47]. Its suppression, by low μM levels of salicylate, in a model of cell death comparable to that induced by reactive oxygen species (ROS) was found. The authors postulate that likely due to suppression of HsGADPH nuclear translocation, mirroring the effect of the anti-Parkinsonian drug Deprenyl.

The same group have also shown [39, 48] that SA targets human high mobility group box 1 (HMGB1), an abundant chromatin associated protein, present in all animal cells; fungi and plants have related proteins. Its diverse effects modulate inflammatory processes. *HMGB1's many activities and receptors likely account for its multiple roles in human disease which include sepsis, arthritis, atherosclerotic plaque formation and cancer.* The effect of SA on HMGB1 occurs at concentrations far lower than those required to inhibit COX enzyme activity; an effect on COX2 is on synthesis rather than activity.

An example of a bacterial homologue enzyme, found in mice, is responsible for synthesis of 2,5DHBA—the iron binding moiety of a mammalian siderophore [49]; that enzyme is a homologue of bacterial EntA which catalyses 2,3DHBA production during enterobactin biosynthesis (Section 5.1). 2,5DHBA can, of course, also derive from the metabolism of SA or benzoic acid.

Other orthologs of a plant SA receptor—NAD(P)-reductase like proteins—have been characterised in the human neuroblastoma SK-N-SH cell line and mouse brain tissue [50]. Their results may point, the authors claim, to the existence of a thermoregulation system that is evolutionary conserved.

5.3.6 ASA and SA as NSAIDs

The few direct studies to validate the assertion 5.3.2a above compared salsalate (which yields only SA on absorption) with SA, generally at the higher doses used in rheumatic diseases. Given what we know about the distribution and relative inhibition of COX1 and COX2 it's not surprising that at comparable doses effects were similar with a predictable lower gastrointestinal toxicity of SA [51]. The authors suggested that, when ASA was originally marketed, commercial forces equated taste and tolerability/toxicity! These prominent rheumatologists concluded that "non-acetylated salicylates should be preferred to ASA in rheumatology". They clearly supported the German proverb:- "Bitter im Mund, gesund im Korper."

While the NSAID categorisation originally served to differentiate the side effects of SA and steroids, very early work had shown a CNS effects specific to salicylates. Later studies—stimulated by discovery of the antipyretic/anti-inflammatory actions of the neuropeptide α-melanocyte stimulating hormone (αMSH)—clearly demonstrated peripheral effects of salicylates introduced into the CNS by injection into the lateral ventricle. These experiments showed that CNS doses which had no effect systemically had a marked effect on the mouse model of inflammation used. The effect was restricted to the salicylates; central injection of an anti-inflammatory dose (when given intra-peritoneally) of indomethacin had no effect: neither did intraventricular dexamethasone or prostaglandin E_2 [43].

More recent work on peroxisome proliferator-activated receptors (PPARs) has also shown the importance of central nervous system actions. Peroxisomes are oxidative (H_2O_2 producing) organelles subserving redox regulation and metabolism of very long chain fatty acids. They are abundant in the CNS, where such fatty acids abound and their increase, when required, is receptor mediated. Studies have compared the anti-inflammatory effect of agonists of PPARα and PPARγ (themselves inactive at the site of inflammation) with the effect of dexamethasone and ASA. Only other agonists and ASA (which itself has generally no direct* PPAR agonist effect) were found to diminish inflammation when given after the inflammatory insult in contrast to the effect of dexamethasone. The conclusion was that PPARα and PPARγ regulate inflammation through a mechanism similar to salicylates and distinct from that ascribed to steroids [53]. The authors postulated that activating PPARs in the CNS could elicit **the release of a salicylate-like compound, an endosalicylate**, which may subsequently cause the release of a physiological anti-inflammatory substance such as αMSH.

These results on CNS activity point to steroid/NSAID differentiation which is at least partially dependent on how agents influence the anti-inflammatory and immunomodulatory effect of melanocortins (ACTH and MSH).

6. Conclusions

Given the ever increasing complexity of SA and ASA effects revealed by basic research it seems blinkered to increasingly restrict focus to platelet/COX effects in the biomedical field.

We reiterate our conclusion that ASA is no mere anti-platelet prototype [54]. That is the case, we aver, for most of the protean pathophysiological effects of ASA and not solely in cancer chemoprevention. The evidence summarised here, particularly our demonstration of the in-vivo synthesis of SA, points to it as a truly endogenous molecule in animals and man. Potential "preserved" receptors which have been described are there, we suggest, not simply to deal with ingested SA or other exogenous precursors.

The place of SA in the biosphere overall is, we think, as pivotal as it appears to be in plants. While a unifying hypothesis to explain its many roles is elusive we suspect they all ultimately relate to the need for evolving life to balance its requirements for oxygen and iron [55]. These authors concluded that the sequestration of iron to restrict its reaction with reactive oxygen species (ROS) is one of our major antioxidant defence mechanisms. They particularly emphasise, in that summary, how such sequestration remains critical to our ongoing resistance to bacterial infection.

The huge increase in energy production arising from enzymatic reduction of oxygen enabled evolution of multicellular animal life. While that was an evolutionary milestone ability to use the resulting reactive oxygen species (ROS) for cell signalling and regulation may have been the first true breakthrough in development of complex life [56]. By then SA was already well established and poised to interact (with ROS) as required. In animals its many effects are unlikely to be less complex than the interactions steadily becoming clarified in plants [39]. *Many may depend upon the type of intricate relationships which initiate plant systemic acquired resistance (SAR) with an initial SA induced redox change. Subsequent SA concentration sensitive*

* However ASA's apparently direct binding to PPARα may explain its stimulation of hippocampal plasticity [52] and potential for prevention of Alzheimers.

oligomer/monomer transformation permits nuclear translocation of a cytosolic messenger to activate immune-associated genes [40].

Re-focus on the importance of the SA moiety of ASA should also lead to further evaluation of SA derivatives which are more active than SA itself in interaction with particular "binding protein/receptors" [47, 48]. At a more basic level we have previously pointed out that, particularly to extend its use in prophylaxis, the risk/benefit profile of ASA may be improved with an SA/ASA combined formulation [54].

Given what we have learned on this investigative journey it is somewhat paradoxical that we embarked upon it driven by desire to capitalise on the hydroxylation of ASA as a biomarker of oxidative "stress" in man—see Section 2.

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Dr. John Robert Paterson (1955–2004), our dear friend who initiated and steered this line of research, always—from schooldays onwards—thought of himself as a chemist first and foremost. Through his long and arduous training in pharmacy, medicinal chemistry, medicine, royal college membership and clinical biochemistry he described himself as a chemist. Most of the work summarised herein, including results published after his death, was either planned by him or arose from discussions during his life. Our prime purpose in undertaking this compilation has been to remain true to his vision. We only pray that there are no glaring chemical errors—the fault is entirely ours if there are.

We are grateful to Hannah Mortlock for the preparation of **Figure 1**.

Conflict of interest

The authors declare no conflict of interest.

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Other declarations

We have done our utmost to accurately reflect the findings and conclusions of the many authors quoted in Section 5. Profuse apologies if our reflections on their studies appear at variance with their interpretations.

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