UNIVERSITY OF STRATHCLYDE

AN INVESTIGATION INTO THE ORIGINS OF SALICYLIC ACID IN MAN

submitted by

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ABSTRACT

Sensitive, accurate and reliable methods to determine the concentrations of salicylic and salicyluric acids in urine and of salicylates in foodstuffs were developed in order to assess the extent of people's exposure to salicylates. These methods involved extraction of acidified aqueous phases with ethyl acetate, high pressure liquid chromatography with systems of stepwise gradient elution, and electrochemical quantification.

Urine from a group of vegetarians who had not taken salicylate drugs contained more of these acids than urine from people with an unrestricted diet. However, the highest amounts of salicylic acid and salicyluric acid were excreted by people who took low doses of aspirin regularly.

Blood from a group of rural Indians contained the highest concentration of salicylic acid.

By examining the blood of normal and "germ-free" rats it was concluded tentatively that the metabolic activity of the gut microflora did not contribute to the amounts of salicylic acid in serum.

Several potential precursors of salicylic acid were investigated as possible dietary sources of salicylates and it was concluded that chorismic acid and benzoic acid might be precursors of salicylic acid in serum.

It was established that food produced from plants cultivated "conventionally" contained less salicylic acid than food produced from plants cultivated under "organic" conditions. Very high contents of salicylates were present in several of the spices that are used in large quantities in Indian cookery.

The evidence presented in this work indicated strongly that people from a rural part of India, who live on highly spiced, principally vegetarian foods, were exposed to salicylic acid to a much greater extent than any of the other groups of people investigated who had not taken salicylate drugs and it is suggested that this extensive exposure might have contributed significantly to the very low incidence of colorectal cancer in this population.

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INTRODUCTION

Salicylic acid (2-hydroxybenzoic acid) is the simplest member of the salicylate family. The salicylates are widely distributed in plants (both angiosperms and gymnosperms) and they occur as salicylic acid (SA) and in the form of related glycosides and carboxyl esters [4]. Extracts of certain plants have been used for centuries to treat inflammatory conditions and it has been established that the salicylates present in these extracts are the source of the anti-inflammatory agent [1]. Saligenin (2-hydroxymethylphenol) and salicin (2-hydroxymethylphenyl-β-D- glucopyranoside) are two salicylates of the bark of the willow that are transformed into SA in man [2]. Ancient Greek and Roman physicians used willow bark for its anti-inflammatory and analgesic properties. In the first century Dioscorides recommended the use of willow bark to treat gout, and its use to treat fever and to alleviate pain during childbirth is mentioned in the Corpus Hippocratian which was completed about 300BC [2].

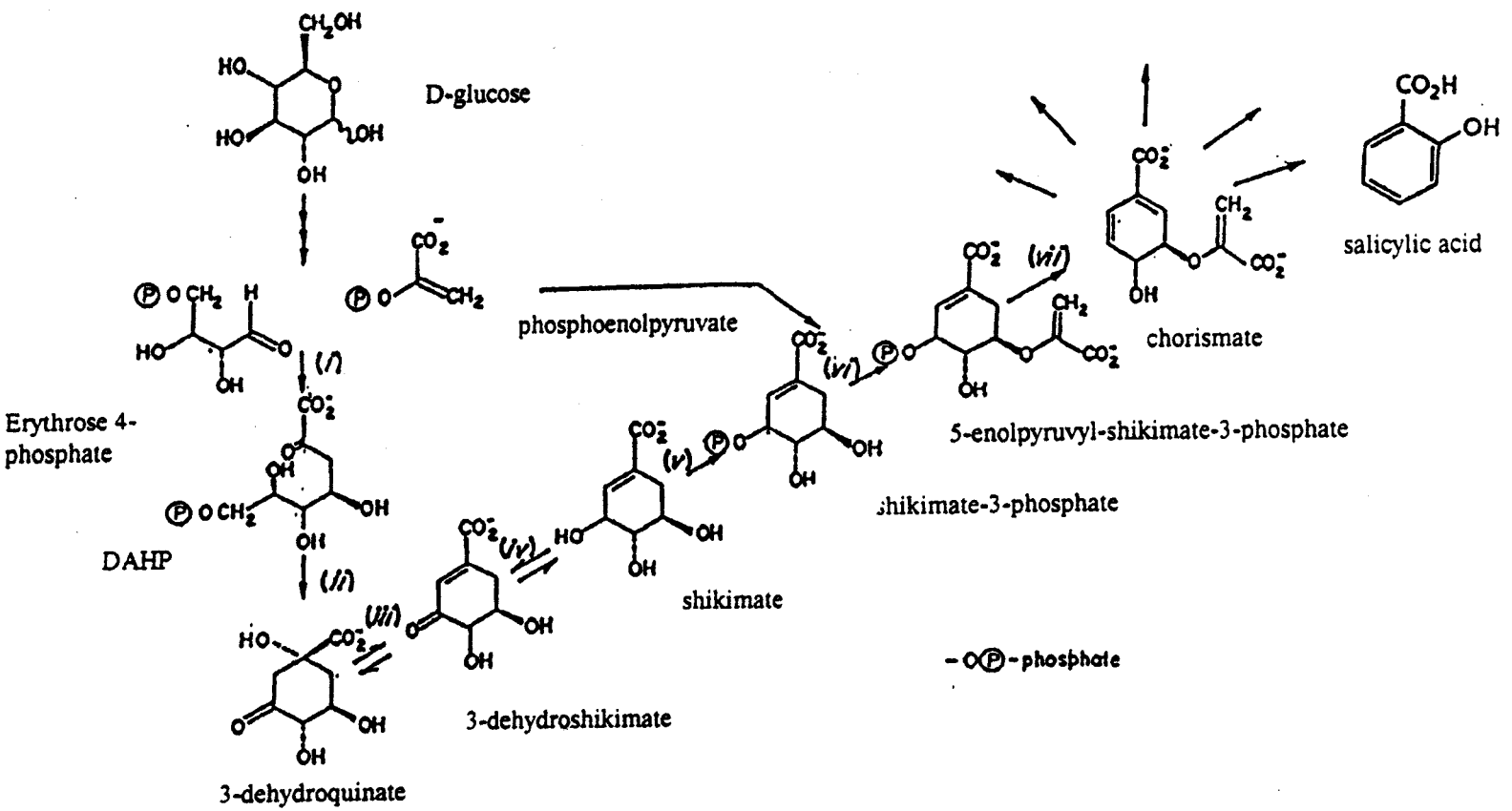
The characterization of salicin took many years and eventually was achieved by Leroux in the mid 1800s after he managed to purify it from meadowsweet. In 1833 Pagenstecher isolated an acid from meadowsweet, which was shown by Meyer in 1870 to be SA [3]. SA was synthesized by the carboxylation of sodium phenoxide by Kolbe in 1852. Subsequently, his method was improved so as to enable the economic production of large quantities of SA, and a factory was established in Dresden [3]. Large doses of bitter-tasting SA were used to treat fever, pain, and inflammation. However, two major problems with taking large doses of SA are vomiting and ulceration of the gut. Although Hoffmann prepared acetylsalicylic acid (aspirin) in 1897 in order to overcome these problems, SA was

used as an anti-inflammatory agent long after aspirin had been produced and sold on a commercial scale.

In plants and bacteria, SA is synthesized by various pathways, including the shikimic acid pathway (Fig.1) and the phenylpropanoid pathway (Fig.2). Shikimic acid was the first of the intermediates of the pathway leading from carbohydrates to the aromatic amino acids to be identified [4]. In this pathway, SA is formed via isochorismate. Most of the SA found in plants, however, appears to be generated by the phenylpropanoid pathway, i.e. via cinnamic acid and benzoic acid [5, 6]. Cinnamate undergoes β-oxidation to form benzoate which then is hydroxylated to give SA. Other synthetic routes to SA in plants and bacteria have been reported, including one in which cinnamate is hydroxylated to give *o*-coumarate, followed by β-oxidation of *o*-coumarate to give SA [7].

In plants, SA is a secondary metabolite that is involved in the initiation of local and systemically acquired resistance to pathogens [8-10]. Plants respond to physical and chemical assault, such as wounding, ultraviolet damage or microbial infection, by initiating both local and systemic actions. Systemically acquired resistance (SAR) is a mechanism whereby the plant recognizes the "threat" at a localized site in its structure so that appropriate defences can be initiated. These defence mechanisms include the transcription of pathogenesis-related genes [11], which enables accelerated cell death protein 6 to act so that damaged cells are removed by apoptosis (programmed cell death) [12 -13]. Apoptosis is a

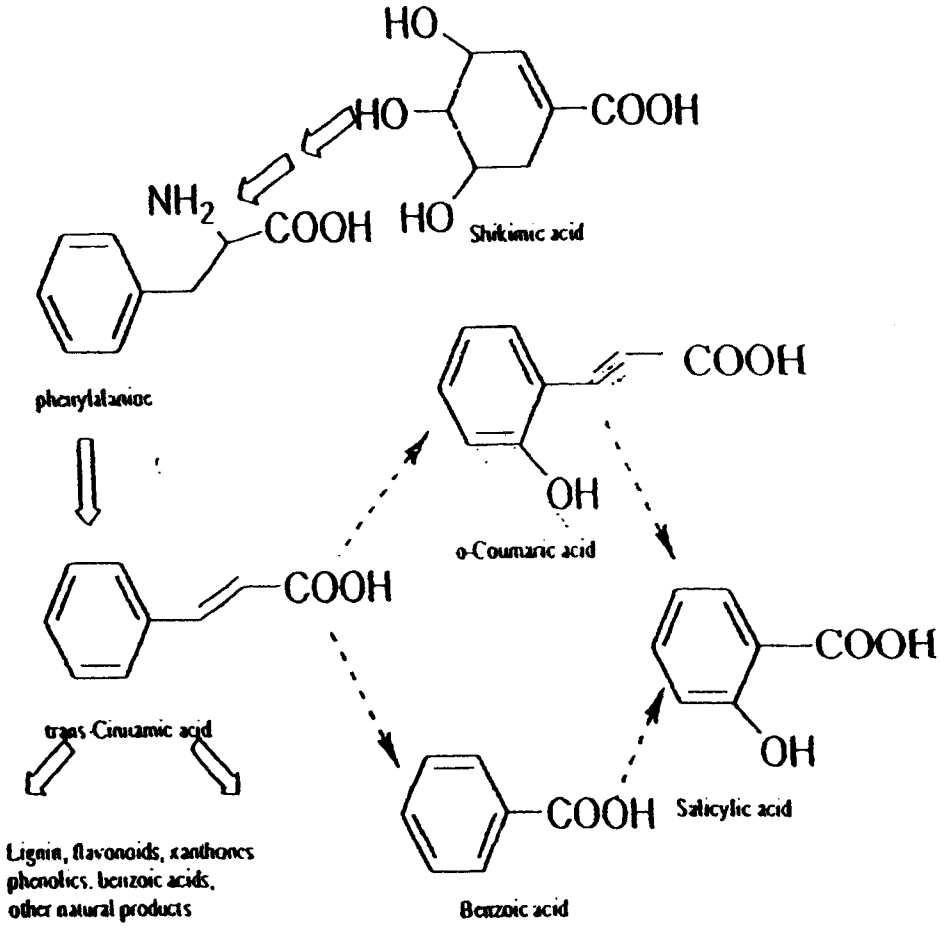
Figure 1. The shikimic acid pathway (adapted from Haslam [4])



DAHP =3-deoxy-D-arabinoheptulosonate 7-phosphate

(i) DAHP synthetase
(ii) 3-Dehydroquinate synthetase (NAD^+, CO^{++})
(iii) 3-Dehydroquinate dehydratase
(iv) 3-Dehydroshikimate reductase (NADPH)
(v) Shikimate synthetase (ATP)
(vi) 5-Enolpyruvylshikimate-3-phosphate synthetase (PHP)
(vii) Chorismate synthetase

Figure 2. The phenylpropanoid pathway (adapted from Raskin [89])



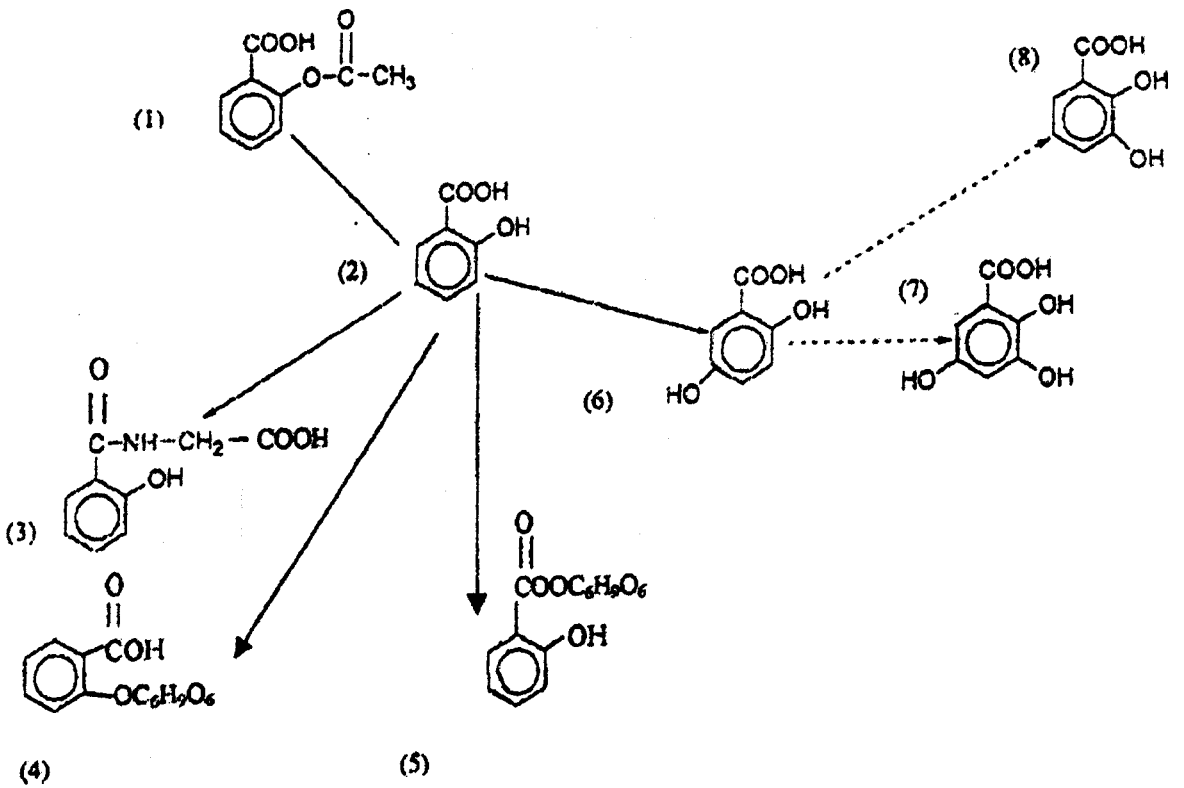
mechanism whereby cell death is achieved in a controlled manner. Ward et al [14] showed that local and systemic resistance were induced by the action of SA on these pathogenesis-related genes. The role of SA in the initiation of SAR also was demonstrated by Vernooij et al [15]. They found that leaves that had developed an enzyme that catalysed the degradation of SA were incapable of initiating the defensive responses, and they suggested that SA was a signalling molecule in SAR. Shulaev et al [16] found that 93% of the SA synthesized by an infected plant was converted into 4-methylsalicylic acid (a more volatile substance than SA) and that the airborne 4-methylsalicylic acid was carried to neighbouring plants to act as a major component of an "alarm system".

SA is involved in several other physiological processes in plants including the regulation of stomatal closure [17], uptake of ions by roots [18], stimulation of flowering [19], and regulation of heat production in *Arum* lilies [20]. Serino [7] found that salicylate induces antibiotic resistance and promotes the uptake of iron in certain bacteria.

Hippuric acid (benzoylaminoacetic acid) is the major urinary metabolite of benzoic acid in man. Starvic and Stoltz [21] reported that quinic acid or shikimic acid taken orally by man caused an increased urinary output of hippuric acid and they showed that intestinal bacteria converted shikimic acid and quinic acid into simple aromatic compounds. It was demonstrated that the intestinal bacteria of rats were responsible for the initial reduction and dehydroxylation of shikimic acid to form cyclohexancarboxylic acid, which then was aromatized within the

animals' tissues [22, 23]. However, the synthesis of SA by higher animals has not been reported. Nevertheless, both SA and its metabolite, salicyluric acid [SU] (Fig.3), might be present in the urine of people who had not taken drugs of the salicylate family. Armstrong et al [24] showed that a substance with the 2-dimensional paper chromatographic characteristics of SU was present in human urine, these workers [24] having examined the urine of 400 people. Their results suggested that many different hydrophobic acidic compounds were present in human urine (which partitioned into ethyl acetate) and they recorded the chromatographic behaviour of 49 of them. They speculated that a considerable number of these compounds, which possibly were phenolic acids, were of dietary origin. Bray et al [25] recognized the difficulty of measuring very small amounts of phenolic acids in urine caused by the fluid's much higher content of much less acidic phenols. These workers [25], by the use of paper chromatography, identified SU in the urine of a rabbit that had been given a dose of SA, and they estimated that the SU excreted was derived from about 5% of the dose administered. In 1963, von Studnitz and colleagues [26] studied the urinary excretion of phenolic acids by people whose dietary intake had been restricted to glucose and citric acid. These workers used paper chromatography and found that several compounds with the chromatographic characteristics of phenolic acids were present in the urine of people who had been taking the restricted diet for three days. They speculated that 10 of the phenolic acids had originated from "endogenous" sources and they suspected that SA was one of these phenolic acids. Young [27] reported the transient appearance of a compound, which he

Figure 3. Metabolism of aspirin in mammals (adapted from Hutt et al [113])



(1) acetylsalicylic acid (aspirin)
(2) salicylic acid
(3) salicyluric acid
(4) phenolic glucuronide of salicylic acid
(5) acyl glucuronide of salicylic acid
(6) 2,5-dihydroxybenzoic acid
(7) 2,3,5-trihydroxybenzoic acid
(8) 2,3-dihydroxybenzoic acid

suspected was SU, in the urine of premature babies. Finnie and co-workers [28] showed that SU was present in the urine of sick children who had not been given salicylate drugs and they established the identity of SU using gas chromatography-mass spectrometry (GCMS). When Ruffin et al [29] investigated the lowest effective administered dose of aspirin that was capable of suppressing the production of human colorectal mucosal prostaglandins (PGs), they reported that SA was present in the plasma of some of their subjects prior to ingestion of aspirin. However, these workers did not explain how they had identified SA. Armstrong et al [24] suggested that one of the compounds they had detected in urine was SA, and they speculated that it might have come from foodstuffs, the action of intestinal bacteria, or might have been formed by the chemical or biochemical transformation of another dietary compound. Also they [24] suggested that the compound suspected of being SA might have been produced by a combination of all of the above mechanisms. In this early work [24 – 28] only Finnie et al [28] characterized the compounds they thought might be salicylates, and none of these groups of workers [24-28] developed methods to quantify them.

By employing high pressure liquid chromatography (HPLC) with electrochemical quantification, Paterson et al [30] demonstrated that SA and two of its hydroxylated metabolites, 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA), were present in the serum of people who had not taken salicylate drugs. These substances had been extracted from strongly acidified serum into ethyl acetate. They tried to establish the identity of SA by using the selectivity of the enzyme salicylate hydroxylase and by using GCMS.

Salicylate hydroxylase catalyses the conversion of SA and closely related *o*-hydroxybenzoates into their corresponding catechols, and it catalyses this process most rapidly with SA as the substrate (Fig. 4).

Animals also develop a variety of substances in response to injury and these include PGs which are local hormones. PGs are synthesized from arachidonic acid, produced by the hydrolysis of membrane phospholipids, by either of two enzymic pathways; one involves lipoxygenase and the other utilizes cyclooxygenase. Cyclooxygenase (COX) is present in cell membranes and it catalyses the oxidation of arachidonic acid to give PGG_2 initially, which then is transformed into PGH_2 (Fig. 5). Non-steroidal anti-inflammatory drugs (NSAIDs), which include SA and its relatives, have been used for decades to alleviate fever, inflammation and pain. In 1971, Vane [31] reported a possible relationship between the effects of NSAIDs and the actions of PGs when he found that NSAIDs inhibited COX and restricted the production of PGs. Subsequently, two isoforms of COX were identified, COX-1 and COX-2. The former, which is a constitutive enzyme of many mammalian tissues [32], is involved in physiological processes that produce PGs so as to maintain vascular and hormonal homeostasis. For instance, COX-1 is involved in the production of PGs that stimulate the secretion of mucus and bicarbonate and repress the secretion of acid in the stomach so as to protect the gastric mucosa. Synthesis of the other isoform, COX-2, is induced in monocytes and macrophages upon stimulation by a variety of factors such as cytokines, mitogens and endotoxins [33-35]; production of COX - 1 is unaffected by these pro-inflammatory agents. One prostaglandin, PGE_2, acts

Figure 4 The conversion of salicylic acid into catechol catalysed by salicylate hydroxylase

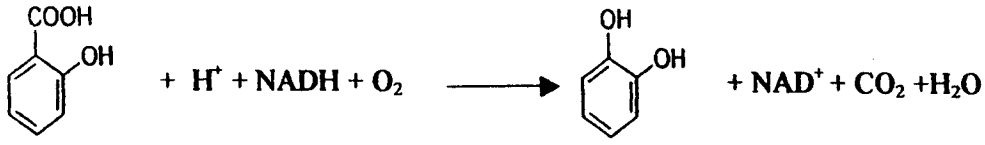
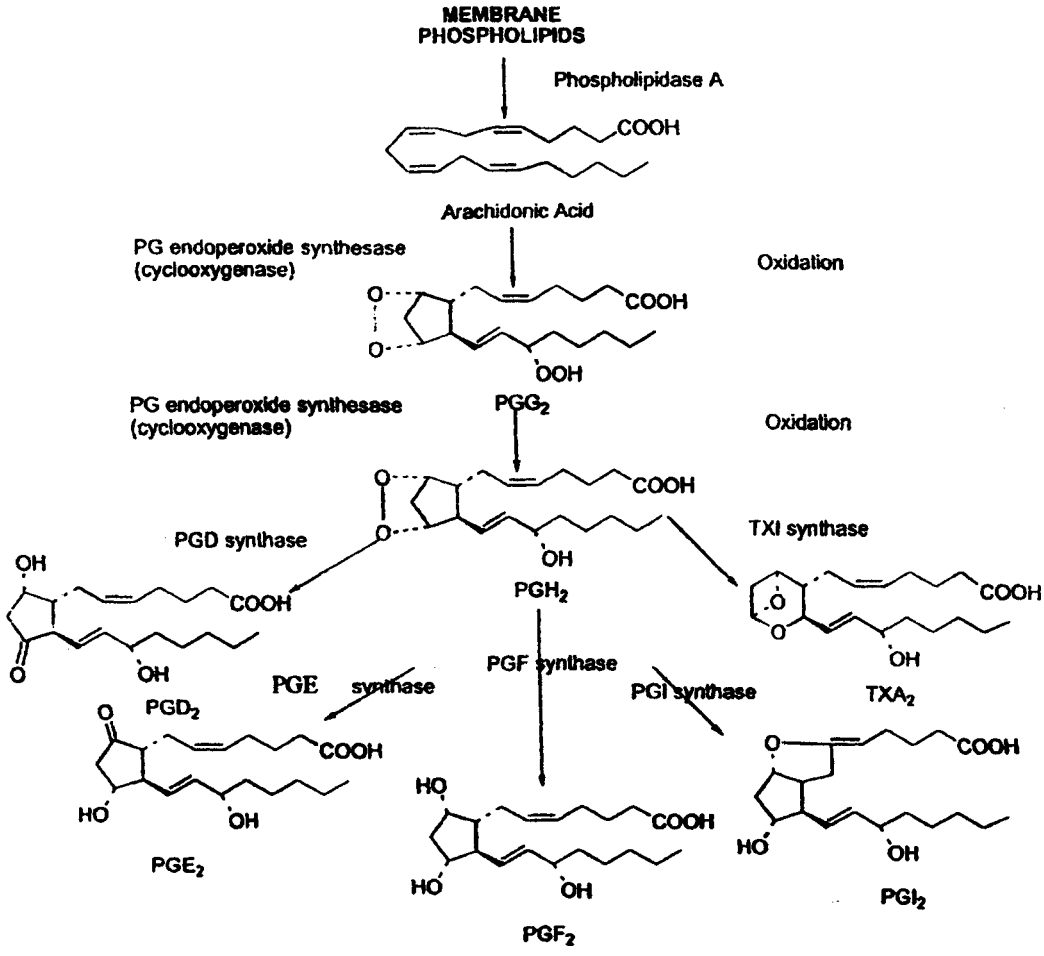


Figure 5. The production of prostaglandins (PGs) from arachidonic acid

(Devlin [100])



on the hypothalamic region of the brain involved in thermoregulation, and it causes increased body temperature resulting in fever (pyrexia) [36]. Several workers have reported that an over-expression of COX-2 is correlated with the occurrence of colorectal cancer [37 - 39]. COX-2 is involved in producing PGs that cause inflammation (by increasing blood flow to the affected area resulting in redness and swelling) and pain (by sensitizing nerve endings). Aspirin inhibits COX-1 and COX- 2 by acetylating a serine side-chain of these isoforms that results in restricted access of substrate to their active sites [40].

Although aspirin and SA are equally effective at suppressing inflammation *in vivo*, it has been found that SA inhibits COX weakly and reversibly [41] and, in a later publication, it was reported that SA was devoid of inhibitory activity towards COX-2 *in vitro* [42]. These observations indicate clearly that the anti-inflammatory property of SA is brought about by a mechanism of suppressing the production of inflammatory PGs that does not involve its direct interaction with COX-2. Recently, a persuasive clue as to how the anti-inflammatory effect of SA is caused has emerged from the work of Wu et al [43] and Xu et al [44]. These authors showed that SA inhibited the transcription of the COX-2 gene at micromolar concentrations (0.1 – 100 μM).

Epidemiological studies also have indicated that people who take aspirin are less likely to suffer a stroke [50] or to develop heart disease [51] than those who do not. People with diabetes have platelets that have an increased tendency to aggregate and they are given aspirin as an anti-clotting agent. Colwell [52], whilst investigating the incidence of myocardial infarctions in people suffering from

diabetes, noted that people who took aspirin suffered fewer myocardial infarctions. C-reactive protein (CRP) is one of a group of plasma proteins whose rate of synthesis in the liver is increased in response to tissue damage. CRP binds to foreign particles, such as bacteria, and causes enhancement of phagocytosis. Over an eight year period, Ridker and colleagues [53] studied 543 men who developed stroke, myocardial infarction or venous thrombosis. Some of them took aspirin over the time course of the study and others did not. The investigators [53] measured the concentrations of CRP (an index of systemic inflammation) in these subjects and they concluded that plasma concentrations of C-reactive protein can be used to predict the likelihood of a future myocardial infarction or stroke. Higher concentrations were correlated with increased risk. They reported also that the consumption of aspirin caused a reduction in the risk of suffering a first myocardial infarction, and that taking aspirin had a far greater effect in those subjects with increased CRP concentrations. They suggested that taking anti-inflammatory agents might help to prevent cardiovascular disease.

Chrubasik et al [54] evaluated the effectiveness of an extract of willow bark (that contained 0.153mg of salicin per mg of solid) to relieve back pain in patients. They confirmed an earlier report [55] that oral administration of a single dose of willow bark extract (that contained 210mg of salicin) relieved back pain, and they suggested that oral doses of the extract may provide an alternative to taking NSAIDs, especially for patients with chronic back pain. The Multicenter Salsalate/Aspirin Comparison Study Group, by using a randomized double-blind parallel-group approach, discovered that SA was as effective as aspirin in the

treatment of rheumatoid arthritis [56].

Colorectal cancer is the second most common cause of death from cancer in the Western world, and in 1996 it accounted for 8.5% of all new cancers [58]. Its incidence in the developed world is higher than in Asia, Central and South America and Africa [59]. A variety of methods and populations have been used in several observational studies, and these have indicated that there is a chemoprotective effect of taking NSAIDs (often aspirin) on the incidence and mortality of colorectal cancers [60, 61]. Smalley and colleagues [62] concluded that people who took NSAIDs were 50% less likely to develop colorectal cancer. In 1983 Waddel and colleagues [63] reported that colorectal polyps underwent regression in patients treated with sulindac, a NSAID. In 1991, Thun et al [64] also reported that there is a positive correlation between taking aspirin and a decrease in the risk of dying from cancer of the colon. From a prospective mortality study of 662,424 adults, who had provided information about how often they took aspirin, they noted that death rates from cancer of the colon in both men and women decreased progressively as the frequency of consumption of aspirin increased. The risk of dying from cancer of the colon among people who were taking aspirin (16 or more doses per month) was approximately half of that among people who did not take aspirin. Schreinemachers and Everson [65] studied 12,668 subjects identified from the National Health and Examination Survey (1971-1975). Some of these people had been taking aspirin regularly prior to interview and others had not. Several years later these subjects were re-investigated to see how many had developed colorectal cancer: 1,257 were found to have developed cancer within 2 years of the initial interview. The incidence of

several cancers, including colonic cancer, was lower in those persons who took aspirin compared with those who had not taken the drug (8.6,11.8% respectively). Schreinemachers and Everson concluded that their observations provided strong evidence of a correlation between taking aspirin and a decrease in the incidence of developing lung cancer, breast cancer and colorectal cancer. The development of a cancer involves many processes such as mutagenesis, cell growth, tumour promotion, immune suppression and metastasis, and PGs are believed to be involved in these processes [45]. Inhibition of the synthesis of PGs by NSAIDs such as aspirin might explain the chemoprotective properties of aspirin [46 - 48, 134, 127].

Evidence that aspirin prevented the development of colorectal cancers also comes from experiments in which laboratory animals were administered a carcinogen and the effects of administration of aspirin on the development of possible tumour precursor states, e.g. aberrant crypt foci (ACF) or tumour itself, were recorded [138]. Generally, administration of aspirin and other NSAIDs caused a decrease in the formation of ACFs and colorectal cancers.

Adenomas are lesions that may develop into cancers. Very recent reports have shown that regular daily consumption of aspirin exerts a chemoprotective effect against the formation and recurrence of colorectal adenomas in man [57, 122, 134]. Baron et al [122] found that a dose of 81mg per day of aspirin taken over several months provided better chemoprotection against developing adenomas than a dose of 300mg. Sandler et al [57] reported the chemoprotective effect of a

regularly taken 325mg dose and Benamouzig et al [133] observed chemoprotective effects produced by doses of 160mg and 300mg. Although various doses of aspirin and different groups of patients were examined, all of these workers found that aspirin afforded protection against the development of adenomas.

Although there is a large body of evidence that shows that aspirin provides a chemoprotective effect against developing colorectal cancer, the mechanisms by which consumption of NSAIDs affords this protection are poorly understood and they are under active investigation [66,67]. However, compelling evidence has been published that reveals that over-expression of COX-2 is correlated with occurrence of colorectal cancer [37 - 39], and this is supported by the results of genetic studies [69]. Several groups of investigators have tried to determine the relationships between doses of NSAIDs and the ability to protect against developing colorectal cancers (see previous paragraph). Krishnan and colleagues [69] attempted to find a reliable biomarker for determining the dose of NSAID that would provide a chemoprotective effect in humans. Two groups of subjects (total number 92) were matched for age ($\pm$ 5 y) and gender with one group being considered to have a much higher risk of developing colorectal cancer. Biopsies (two per subject) of the distal sigmoid colon were obtained from these two groups of subjects. One biopsy was removed prior to taking aspirin and the other was obtained 29 days after the subjects had started taking aspirin (81mg per day); the concentrations of PGE_2 in the tissues were measured. Krishnan et al found that taking 81mg of aspirin per day reduced the concentrations of PGE_2 by 65% (in the

group considered to be at normal risk) and by 70% (in the group considered to be at higher risk).

Many of the investigations into the biochemical/pharmacological mechanisms whereby NSAIDs prevent the development of colorectal cancers have involved treatment of cells in suspension with "solutions of aspirin". A saturated solution of aspirin in water at 25 °C has a concentration of about 18mM [70]. However, as soon as dissolution of aspirin in an aqueous solvent is attempted the phenolic ester starts to degrade. In an aqueous solution the substance is most stable at pH 2-3, and even at this pH it decomposes [70]. If the pH of the solution is adjusted to <2 or >3 the rate of decomposition is magnified massively [70]. Yoshikawa et al [71] claimed that treating colorectal adenocarcinoma cells with a "solution of aspirin" induced cell death via apoptosis. However, they used a previously prepared "solution of aspirin" that had been stored for an undisclosed period of time. Then it was diluted with 15mMTris/HCl buffer to adjust its pH to 7.0 before it was added to the cells and the mixture was incubated at 37°C for 5h or more. Examination of these experimental conditions reveals that their claim to have treated the cells with aspirin is naïve. Moreover, the cells will certainly have contained esterases, whose distribution within animal tissues is ubiquitous [72]. Non-specific esterases catalyse the hydrolysis of aspirin [73]. Osnes et al [74] investigated the effect of aspirin and sodium salicylate on the concentrations of lipopolysaccharide-induced tissue factor (TF), tumour necrosis factor (TNF_α) and PGE_2 produced by human monocytes. In order to minimize the degradation of aspirin they kept its stock solution at pH 3– 4. However, because the cells were unable to tolerate acidic pH, they adjusted the pH of the suspending medium to

pH 7.4 immediately after addition of the "solution of aspirin" to the cells. Therefore, it is reasonable to conclude that the effects reported by Yokishawa et al [71] and ascribed to aspirin are most likely to have been brought about by SA, the product of aspirin's hydrolytic decomposition. Even under the more carefully considered conditions employed by Osnes et al [74] it is entirely uncertain to what extent, if any, the effects they reported were due to aspirin. It is entirely possible that these effects also were due to the action of SA.

Most of the salicylates that are absorbed by higher animals are derived from medicines (such as aspirin) and foodstuffs (to which they are also added as preservatives and flavouring agents). Although the use of SA as a food preservative has been prohibited in France since the 1880s and in the USA since 1904, it is incorporated into many topically-applied medicines as a non-toxic antiseptic and as a preservative [2]. In the USA, the addition of benzoic acid (a possible precursor of SA) to foodstuffs in quantities exceeding 0.1% is prohibited under the Federal Food, Drug and Cosmetic Act. SA is present and has been quantified in many foodstuffs [75,76], the highest concentrations appearing in certain spices, herbs, fruits, and vegetables [77 - 79]. Janssen et al [80] determined that the "total average salicylate" excreted in urine of people who had a wide variety of diets was about 10 μmol per 24h (range, 4– 34 μmol per 24h). Janssen et al also estimated that strictly vegetarian diets provided <43 μmol (6mg) of salicylates per day and they speculated that these amounts were too low to affect the risk of developing coronary heart disease or colorectal cancer. Janssen et al gave no information about the salicylates they had included as "total salicylates"

nor did they describe how they had confirmed the identities of these compounds [80].

Many students of the interrelationships between disease, health and diet have suggested that there is to be gained a benefit to health by eating fresh fruit and vegetables [81]. Indeed, the World Health Organization recommends a daily intake of "5 portions of fruit and vegetables" because this amount is beneficial to health and seems to provide protection against conditions such as cardiovascular disease and some cancers [82]. It has been suggested that the fibre, the antioxidant and the vitamin contents of fruits and vegetables are involved in endowing these beneficial effects. Eastwood [83] suggested that the secondary plant metabolites, such as alkaloids and phenols, in the diet might be responsible.

The variation in incidence of colorectal cancer throughout the world has led to speculation that different diets, gut bacteria and environmental conditions are factors which may be of etiological significance. A low incidence of colorectal cancers has been noted in India and there is regional variation in the low incidence of colorectal cancer in this country; there is an even lower incidence in rural areas than in neighbouring urban areas [84,137]. Immigrants from India living in Singapore and New South Wales were found to have a lower incidence of colorectal cancers than the local population but a higher incidence than people living in India [85,86]. All of these findings suggest that diet may make an important contribution to the incidence of colorectal cancer and that the global variation is not necessarily due to the better diagnostic techniques of the developed world or of urban areas compared to those of rural areas. In rural India,

the incidence of colorectal cancer is extremely low and the diet of rural Indians is predominantly vegetarian [87]. The main components of their diet are fruits, vegetables and cereals flavoured with substantial quantities of herbs and spices (which are known to contain significant amounts of SA). Most of these foodstuffs will have been prepared from plants that have been grown without the application of pesticides, fungicides or herbicides. SA is a defence hormone of plants and it is likely, therefore, that these plants will contain increased amounts of SA. A diet that is rich in SA might account for the very low incidence of colorectal cancer in this part of the world.

Blacklock et al [88] determined and compared the concentrations of SA in sera from three groups of people; a group of patients taking low-dose aspirin (75mg/day), vegetarians and non-vegetarians who had an unrestricted diet. The vegetarians and the non-vegetarians had not taken salicylate drugs. Higher concentrations of SA were found in the sera of the group of patients taking low-dose aspirin (median concentration, 10.03 μmol/l; range, 0.23-25.4 μmol/l) and in the group of vegetarians (median concentration, 0.11μmol/l; range, 0.04-2.47 μmol/l) than in the non-vegetarians (median concentration 0.07 μmol/l; range, 0.02-0.20 μmol/l). An overlap in the range of concentrations of SA in sera from the vegetarians with those in sera from the patients taking low-dose aspirin was observed. This overlap suggests that a diet containing a substantial proportion of fruit and vegetables might contribute sufficient SA to produce a serum

concentration of SA that could afford a benefit to health. As stated earlier, a diet rich in fruit and vegetables, and, therefore, perhaps rich in SA, appears to be beneficial to health [81] and to provide protection against cancer of the colon and against cardiovascular disease [82]. The results obtained from the determination of the concentrations of SA in sera and/or urine may provide a valuable insight into the links between the phenolic acid, health and disease.

Paterson et al [30] suggested a possible dietary source for the SA found in sera of people who had not taken salicylate drugs. Other source(s) of these salicylates have not been investigated and any investigation of alternative sources must include a consideration of the diet because many foodstuffs contain salicylates. Studies designed to investigate the potential contribution of the actions of gut bacteria are required also, since certain enteric bacteria are capable of transforming compounds into SA and other bacteria are capable of dissimilating SA [89,90]. Consideration must also be given to the possibility that the people themselves might be capable of transforming compounds found in their diet into SA. Various biosynthetic pathways leading to SA have been identified in plants and bacteria. Perhaps the SA found in sera from people who do not take salicylate drugs might be derived from compounds such as benzoic acid, *o*-coumaric acid or chorismic acid that originate from dietary plant materials. These substances then might be converted into SA.

The measurement of the concentrations of salicylates in people with a variety of

diets may provide useful information concerning the effects of dietary salicylates on various disease states. However, to do so, it will be necessary to develop sensitive, precise and reliable methods to identify and quantify the various salicylates that are found in low concentrations in man. Also it would be helpful to develop methods that enable the determination of these salicylates in urine, collection of which does not involve an invasive procedure. Urine collected over a 24h period would provide a means of examining the exposure to salicylates over a longer time-averaged scale. Determination of the salicylates in a sample of blood provides a measurement of the concentration of salicylates at the time of removing the blood, and this value may be strongly affected by various factors including renal function, urinary pH and diet. The information obtained by examining urine collected over 24h would provide more informative data concerning people's exposure to SA. Knowing that many foods, especially those derived from plant materials, contain SA, it might be possible to design experiments to investigate the relationship between the contribution made by diet and the amount of salicylates excreted.

The aims of this project were to characterize and quantify the salicylates present in the urine of people who do not take salicylate drugs and to investigate the possible sources of these compounds. This work involved identifying the salicylates of urine by investigating their susceptibility to selective enzymic transformation and by using GCMS. In addition, precise and reliable methods of determining the concentrations of these salicylates were developed and the values observed in urine

from various groups of people with a variety of diets were compared.

Certain enteric bacteria synthesize and dissimilate SA, and they might have generated some of the SA in individuals who had not taken salicylate drugs. An investigation of the concentrations of SA in serum from two groups of rats (one group with, and the other group without established intestinal microflora) might provide a way of investigating the contributions of gut bacteria to the concentrations of SA observed.

In addition, a study of the concentrations of SA in sera from a wide variety of animals might indicate the extent to which salicylates occur in blood found throughout the Animal Kingdom.

Finally, an investigation of the contribution of possible dietary precursors of SA (*o*-coumaric acid, chorismic acid and benzoic acid) to the concentrations of SA in sera was undertaken. Benzoic acid is used to preserve many foods [129] and it occurs naturally in many plants [5,6]. How ingestion of benzoic acid might affect the concentration of salicylates in the blood and urine of people who do not take salicylate drugs was investigated.

The results of the work proposed should provide valuable information about the source(s) of the salicylates that are found in people who do not take salicylate drugs and the extent to which they are exposed to these substances.

2.0 MATERIALS AND METHODS

2.1 Materials

From Sigma Chemical Co. Ltd., Poole, UK

Salicylic acid, salicyluric acid, 4-methylsalicylic acid, glacial acetic acid, acetyl chloride, salicylate hydroxylase, trisodium citrate, methyl salicylate, tripotassium ethylenediaminetetraacetate (EDTA), hydrochloric acid, *o*-coumaric acid, chorismic acid and sodium hydroxide.

From Presearch, Hitchin, UK

Diamond paste.

From Rathburn Chemicals, Walkerburn, UK

Water, methanol, acetone, ethyl acetate (all HPLC grade).

2.2 Food, blood or blood derivatives, and groups of people studied

2.2.1 Blood or derivatives from non-human animals

Blood, serum or plasma, were obtained from animals at London Zoo or the Department of Biological Services, University of Glasgow. Blood was collected according to the approved codes of practice of these institutions. Some mice were treated with antibiotics (neomycin, 100 mg/kg/day) for 4 days prior to collection of blood. Other samples of serum from animals were purchased from Charles River UK Ltd., Margate, Kent.

2.2.2. Food

Soups, produced by a variety of manufacturers, were bought from local retailers. Other soups were prepared in colleagues' homes. A total of 45 soups were examined. A full list of the soups examined is shown in Table XIV.

Cooked foods were obtained from Safeway Food Store, Dumfries and The Jewel in the Crown, Indian Restaurant, Dumfries

A selection of spices (n = 14) were obtained from a retailer in Glasgow who supplies the local Indian/Pakistani community. Also, samples of some of these spices were purchased from a health food retailer. Tamarind, asafoetida and fennel, imported from New Delhi, were from Dr R. Srivastava.

A selection of wines (n=17), produced from grapes grown under "organic" conditions or "conventionally" were examined for their content of salicylic acid. Two of the wines were produced from grapes that had been infected with *Botrytis* and one wine was produced from late harvested grapes.

2.2.3 Blood or urine from different groups of people

Ethical approval was obtained to collect blood and urine from human volunteers.

a. People not receiving aspirin

Blood and /or urine was obtained from three groups of people and two individuals. One group had been recruited during an earlier study [88] and had donated samples of blood and urine. The urine had been collected over a 24h

period [88]. These people were in voluntary retreat at the Samye-Ling Buddhist Community, Eskdalemuir; they had not taken aspirin and had a strict vegetarian diet (n = 21; 15 male, 6 female, median age 43.5 years, range, 21 – 73 years). Serum was separated from blood as described in section 2.3.1. The serum and urine were stored at –70°C prior to examination.

A second group of people, who donated urine collected over a 24h period, was recruited from employees of Dumfries and Galloway Royal Infirmary. These people (n = 27; 10 male, 17 female, median age 36 years, range 23 – 56 years) had an unrestricted diet and had not taken aspirin.

An individual (male, aged 45 years) supplied urine that had been collected over six consecutive 12 hourly intervals. Samples of blood were withdrawn from him at 12 hourly intervals throughout this time period. During these three days he consumed only milk and water and he had not taken salicylate drugs during the previous fortnight.

Samples of blood were withdrawn from two individuals (males, 45 and 56 years) at 24 hourly intervals over a period of four days. They also supplied urine (collected over 24 hourly intervals) throughout this four day time period. Both of them had eaten the same types of food in the same quantities throughout the four days. On the third day one of them received orally 2.0g of sodium benzoate (two 0.5g capsules twice daily) and the other took 1.0g of sodium benzoate (one 0.5g capsule twice daily). Neither had taken salicylate drugs during the previous fortnight.

Blood and urine was obtained (at approx. 1hourly intervals) from a male volunteer (male, aged 45 years) who had fasted for 10h and who had not taken salicylate drugs during the previous 2 weeks. The volunteer then consumed a portion of cooked vegetables that contained 94.03 mg of "total salicylate".

Samples of blood were obtained from a group of people living in a rural area near Chennai (Madras), India (n = 21; 10 male, 11 female. median age 32 years, range 23 – 51 years). They had unrestricted, traditional diets and had not taken salicylate drugs.

b. People in receipt of aspirin

Two groups of people were recruited from local general medical practices in Dumfries and Galloway. One group (n = 15; 5 male, 10 female, median age 61 years, range 46 – 71 years), had taken low-dose aspirin (75 mg/day) for more than two months. The other group (n = 25; 22 male, 3 female, median age 66 years, range 49 – 76 years) had taken 150mg of aspirin /day for more than two months.

2.3 Methods

2.3.1 Collection and treatment of blood.

Human blood was withdrawn from the anticubital vein by using plastic serum gel blood collection tubes fitted with a FG 20 or 21 gauge needle (Sarstedt, Leicester, UK). After centrifuging (2000g for 10 min at 4°C), the serum was stored in plastic vials at -70°C until required.

Sera from animals were stored in portions at -20°C until required. The blood from animals was centrifuged (2000g for 10 min at 4°C) and the sera obtained was stored in plastic vials at –70°C until required.

2.3.2. Treatment of urine

The volumes of urine collected over 12h or 24h periods were recorded. After the urine was centrifuged at 2,000g for 10 min at 4°C it was divided into portions (1ml) which were stored at -70°C until required.

2.3.3. Treatment of soup and spices

Portions of the soups (2 x 40 ml) were homogenized for 5min using an Ultra-Turrax homogeniser (Janke and Kunkel, Slauden, Germany). The homogenised soups were stored in 1g lots at -20°C until required. Portions of spices were ground to produce fine powders. Portions (50mg) of the powders were stored at -20°C until required.

2.3.4. Extraction of materials from food, plasma, sera and urine

To portions (0.5 ml) of plasma, urine, sera, or wine were added 55.0 µl of solutions of internal standard (4-methylsalicylic acid) to give final concentrations of 2µmol/l in sera, wine and plasma or 20µmol/l in urine, 5.5µl of a solution of EDTA to give final concentrations of 100µmol/l and 1 mol/l hydrochloric acid (so as to adjust the pH to 2.0). Ethyl acetate (2ml) was added and the mixture was vortexed for 15 min. After centrifuging (2,000g for 10 min at 4 °C) the organic layer was removed and the aqueous layer was re-extracted with a further 2ml of ethyl acetate. The organic layer was removed and the combined extracts were evaporated to dryness at 70°C with a stream of nitrogen.

To portions of homogenized soup (1g) were added 55 µl of a solution of internal standard (4-methylsalicylic acid) to give a final concentration of 2µmol/l, 5.5µl of a solution of EDTA to give a final concentration of 100µmol/l and 1 mol/l hydrochloric acid (so as to adjust the pH to 2.0). Ethyl acetate (2ml) was added and the mixture was vortexed for 15 min. After centrifuging (2,000g for 10 min at 4 °C) the organic layer was removed and the aqueous layer was re-extracted with a further 2ml of ethyl acetate. The organic layer was removed and the combined extracts were evaporated to dryness at 70°C with a stream of nitrogen.

To portions of powdered spice (50mg) were added 3ml of 0.1M HCl, 55 µl of a solution of internal standard (4-methylsalicylic) acid to give a final concentration of 2µmol/l, 8.25µl of a solution of EDTA to give a final concentration of 100µmol/l and ethyl acetate (3ml). The mixtures were vortexed for 15 min and centrifuged (2,000g for 10 min at 4 °C). The organic layer was removed and the aqueous layer was re-extracted with a further 3ml of ethyl acetate. The organic layer was removed and the combined extracts were evaporated to dryness at 70°C with a stream of nitrogen.

To determine the content of "total" salicylates in spices, portions of powdered spices (50mg) were suspended in 3ml of 2.5M NaOH. After 24h at room temperature, hydrochloric acid (1ml of 5.3M) was added to adjust the mixtures so that they contained a final concentration of 0.1M HCl. To these mixtures were added 55 µl of a solution of internal standard (4-methylsalicylic acid) to give a

final concentration of 2µmol/l and 5.5µl of a solution of EDTA to give a final concentration of 100µmol/l. The mixtures then were vortexed for 15 min and centrifuged (2,000g for 10 min at 4 °C). The organic layer was removed and the aqueous layer was re- extracted with a further 3ml of ethyl acetate. The combined extracts were evaporated to dryness at 70°C with a stream of nitrogen.

2.3.5 Chromatographic separation of the substances

The compositions of the chromatographic mobile phases were as follows.

Mobile phases for the examination of extracts of food and sera

Mobile phase A	30mmol/l citrate (pH adjusted to 4.0 with acetic acid) containing 50% methanol (v/v)
Mobile phase B	30mmol/l citrate (pH adjusted to 3.8 with acetic acid) containing 5% methanol (v/v)

Mobile phases for the examination of extracts of urine

Mobile phase A	30mmol/l citrate (pH adjusted to 4.0 with acetic acid) containing 50% methanol (v/v)
Mobile phase C	30mmol/l citrate (pH adjusted to 3.8 with acetic acid)

The mobile phases were prepared daily as required. They were filtered through a 22µm Magna nylon filter (Sigma) prior to degassing using a Jour X Act 4-channel degasser (Presearch, Hitchin, UK).

The dried extracts of food or serum were reconstituted in a mixture of 0.5ml of Mobile Phase B and 5.5µl of EDTA (10mM) by vortexing for 5 min. The combined dried extracts of urine were reconstituted in a mixture of 0.5 ml of EDTA (100µmol/l) and 5.5µl of EDTA (10mM) by vortexing for 5 min.

Portions (50µl) of the reconstituted extracts were applied to the chromatographic column. Elution was accomplished using a pump (PU980, Jasco, Gt. Dunmow, UK) and a ternary gradient unit (LG 980 - 02), Jasco, Gt. Dunmow, UK) using the programmes of stepwise elution described in Tables I and II.

The substances eluted were detected electrochemically (Antec Decade detector, Presearch, Hitchin, UK) at an oxidation potential of +1.1 V [30]. The detector temperature was maintained at 30°C. The glassy carbon electrode was polished every 6 weeks with diamond paste and a silk cloth. In addition, the glassy carbon electrode was cleaned with methanol, and the solution of potassium chloride (3.5M) in the silver/silver chloride reference electrode was replaced daily.

2.3.6. Calibration of the methods for determining SA and SU

Concentrated solutions of SA (20µmol/l), chorismic acid (20µmol/l), SU (200µmol/l), and the internal standard (4-methylsalicylic acid, 20µmol/l) were prepared with double-distilled water and heated at 60°C for 1h. Sets of aqueous solutions containing internal standard (2.0µmol/l) and EDTA (final concentration, 100µmol/l) and the determinands in the concentration range 0.02 –200 µmol/l were prepared also (see Table III).

Portions (50µl) of these aqueous solutions were applied to the HPLC column and the phenolic acids were separated using the programmes of stepwise elution described in Tables I and II.

Table I - Programme of stepwise elution for the separation of salicylic acid in extracts of food and blood.

The rate of elution was 0.5ml/min

	Composition of eluent (v/v)	
Time of elution (min)	% Mobile Phase A	% Mobile Phase B
00.00 - 05.00	25	75
05.01 - 28.00	0	100
28.01 - 43.00	100	0
43.01 - 59.00	25	75

Table II - Programme of stepwise elution for the separation of salicylic acid and salicyluric acid in extracts of urine

Time of elution (min)	Composition of eluent (v/v) % Mobile Phase A	% Mobile Phase C (v/v)	Rate of Elution (ml/min)
00.00-02.00	50	50	0.5
02.10-03.00	40	60	0.5
03.10-04.00	30	70	0.5
04.10-05.00	20	80	0.5
05.10-06.00	10	90	0.5
06.10-10 00	5	95	0.3
10.10-15.00	0	100	0.3
15.10-25.00	100	0	0.5
25.10-45.00	100	0	0.3
45.10-59.00	50	50	0.5

Table III – Concentrations of salicylic acid (SA), chorismic acid (CA) and salicyluric acid (SU) in aqueous solutions used for calibration

SA (μmol/l)	0.02	0.20	2.00	10.00	20.00	-	-
CA (μmol/l)	0.02	0.20	2.00	10.00	20.00	-	-
SU (μmol/l)	-	0.20	2.00	10.00	20.00	100.00	200.00

These solutions also contained 4-methylsalicylic acid (2μmol/l or 20μmol/l) and EDTA (final concentration, 100 μmol/l).

Calibration graphs (plots of the ratio of the detector response due to the determinand to that due to 4-methylsalicylic acid against concentration) were constructed for SA, chorismic acid and SU.

2.3.7. The identification of SA and SU

a. Use of salicylate hydroxylase

Salicylate hydroxylase is an enzyme that catalyses the conversion of congeners of SA into catechols (Fig. 4). This enzyme has restricted substrate specificity [91] and its catalytic activity was used to try to identify SA in extracts of soup and foodstuffs and SA and SU in extracts of urine.

To dried extracts of foodstuffs and urine and to aqueous solutions containing SA (1.0 μmol/l) or SU (1.0 μmol/l) were added potassium phosphate buffer (final concentration, 0.03 mol/l; pH 7.62), salicylate hydroxylase (0.4 mg in 0.5 ml of phosphate buffer) and NADH (final concentration, 146 μmol/l) in a final volume of 1.5ml. Reaction mixtures devoid of salicylate hydroxylase served as controls. The reaction mixtures were shaken at 30°C for 15 min and the pH of the mixtures was adjusted to 2.0 using 1mol/l HCl. The mixtures then were extracted with ethyl acetate as described in section 2.3.4. Portions (50μl) of the reconstituted extracts were applied to the chromatographic column as described in sections 2.3.5 and 2.3.6.

b. Variation of chromatographic conditions

Extracts of foodstuffs or urine were prepared and the reconstituted

extracts were applied to the chromatographic column.

i. Chromatography of extracts of food.

Reconstituted extracts of foodstuffs and solutions containing SA and internal standard were chromatographed using isocratic elution with mobile phase A at a rate of 0.5 ml/min.

ii Chromatography of extracts of urine

Reconstituted extracts of urine and solutions of SA and of SU were chromatographed using a programme of stepwise elution as described in Table II using mobile phase A1 in place of mobile phase A. Mobile phase A1 was 30mmol/l citrate (adjusted to pH 4.0 with acetic acid) containing 65% v/v methanol i.e. mobile phase A1 contained 15% more methanol than mobile phase A.

c. Gas chromatography-mass spectrometry

A portion (5 ml) of urine (subsequently found to contain 0.84 μmol/l of SA and 5.56 μmol/l of SU), a portion of soup (5g) [subsequently found to contain 0.241 μmol/l (16ng/g) of SA], and portions of spices (50mg) were extracted with ethyl acetate as described previously but without the addition of internal standard. The dried extracts (of soup or spices) were dissolved in 0.5 ml of mobile phase B. The dried extracts of urine were dissolved in 0.5 ml of 100μmol/l EDTA. Portions (50 μl) of the reconstituted extracts were applied to the HPLC column and elution was accomplished according to the programmes described in section 2.3.5. The electrochemical detector was switched off in order to prevent the oxidation of the

substances eluted. After application of extracts of urine, the fraction eluted between 1 min prior to the R_t of SU and 1 min after the R_t of SA was collected. After application of extracts of soups and spices the fraction eluted between 1 min before and 1 min after the R_t of SA was collected. The fractions collected were acidified and extracted with ethyl acetate (2 ml) and the organic solvent was removed as described previously. A solution of SA (200 µmol/l), SU (200 µmol/), and the dried extracts were treated with 2 ml of acetyl chloride in methanol (300 µl of acetyl chloride added to 10 ml of methanol containing 0.1% water). The mixtures were heated at 60°C for 4 h and then were dried at 70°C with a stream of N_2. The residues were dissolved in 2 ml of ethyl acetate and portions (1 µl) were applied to a GC DB-5 capillary column (30m x 0.25 mm) fitted to a Fisons 8000 series gas chromatograph interfaced with a MD 800 mass spectrometer (Thermoquest, Manchester, UK). The temperature of the column was increased from 54° C at 11° C / min until it reached 300° C. The injection port temperature was 200° C and the mass spectrometer electron energy was +70 eV set in full scan mode. The R_t of the derivatives of SA and SU formed by treatment with acetyl chloride in methanol were compared with those derived from the substances separated from extracts of spice, urine and soup. The total ion chromatograms of these compounds were compared with those of the methyl esters of SA and SU contained in the Pfleger, Maurer and Weber library [92] and with the total ion chromatogram generated when 1µl of a solution of methyl salicylate (200 µM in ethyl acetate) was applied to the GCMS under the same conditions.

2.3.8. The characteristics and reliability of the methods developed for the determination of SA and SU.

a. Precision of the method for the determination of SA in soup and of SA and SU in urine

i. Urine

The intra-assay coefficient of variation (CV) was determined by measuring the concentrations of SA and SU in separate extracts of the same urine prepared at 8 different times within a period of 24 h. Inter-assay precision was determined by measuring the concentrations of SA and SU in another specimen of urine on 8 occasions within a period of 10 days (a separate extract of urine was prepared on each occasion).

ii. Soup

The intra-assay CV for the determination of SA was calculated from duplicate determinations of the content of 22 soups. The inter-assay CV for the determination of SA in four soups was also determined. (The contents of two soups were determined on four separate occasions and the contents of the others were determined five times).

b. Limit of detection of the method to determine SA and SU in urine

The limit of detection of the method was estimated by determining the concentration of an aqueous solution of SA or SU that produced a signal with an amplitude that was three times that of average "baseline noise" of the chromatogram

at the R_t of the determinands [93]. Chromatograms were generated for solutions containing SA or SU at the concentrations used for calibration and the amplitude of the baseline noise was assessed from these chromatograms at the R_t of the determinands.

c. Stability of SA and SU in urine

The stability of the SA and SU present in urine was investigated by determining their concentrations in freshly collected urine and in this urine after it had been stored for 24 h at room temperature, at 4°C and at –20°C.

d. Efficiency of extraction of SA and SU into ethyl acetate

i. From urine

To portions of urine (0.5ml) were added SA and SU (to final concentrations of 0.05, 1.0 and 2.0 μmol/l) and EDTA (to a final concentration of 100μmol/l). The mixtures were adjusted to pH 2 with hydrochloric acid and the phenolic acids were extracted into ethyl acetate as described in section 2.3.4. Portions (50μl) of the dried reconstituted extracts were applied to the chromatographic column.

Portions of the urine also were extracted with ethyl acetate to determine the concentrations of SA and SU prior to the addition of the known concentrations of the phenolic acids.

ii. From soup

To portions of soup (1g) were added SA (15.2ng) and EDTA (to a final

concentration of 100μmol/l). The mixtures were adjusted to pH 2.0 with hydrochloric acid and the SA was extracted into ethyl acetate as described in section 2.3.4. Portions (50μl) of the dried reconstituted extracts were applied to the chromatographic column. Portions of the soup also were extracted with ethyl acetate to determine the concentration of SA prior to the addition of the known concentrations of the SA.

2.3.9 Numerical methods

a. Statistical parameters

Data are shown as mean values and their coefficients of variation (CV) when the individual values were distributed normally, or as median values and the range of values observed when the individual values were not distributed normally. The significance of the difference between the medians of sets of observations was assessed using the Mann-Whitney U-test [94,95]. The quality of least squares linear fits was assessed from values of the correlation coefficient (r). The calculations were performed using a Gateway EV700 computer equipped with Excel (Microsoft) and Argus (Microsoft) statistical software.

b. Chromatographic resolution

The resolution factor (R) is a parameter that describes the degree of chromatographic separation of two compounds. R was calculated using equation 1 where V_1 and V_2 are the retention times of the compounds and W_1 and W_2 are the widths of their peaks at baseline. A value of $R \geq 1.0$ indicates that the compounds have been separated completely.

$$R = \frac{V_2 - V_1}{0.5\,(W_1 + W_2)}$$

Equation 1

3.0 RESULTS

3.1 Development of a method for determining the concentrations of SA and SU in urine.

3.1.1 Chromatographic separation of SA, SU and the internal standard

SA and two of its metabolites, 2,3 dihydroxybenzoic acid and 2,5 dihydroxybenzoic acid, have been found in the sera of people who had not taken salicylate drugs and their concentrations have been determined [30]. SA is extensively metabolized in man (Fig. 3) and its conjugation with glycine occurs in the kidney. The development of a method to determine the concentrations of SA and SU in urine is, therefore, essential to obtain information about the exposure of man to salicylates.

Several characteristics need to be incorporated into a method of determining SA and SU that requires their prior chromatographic separation from complicated mixtures. These include reasonably short R_ss for both internal standard and determinands, clean separations of these substances, and their well defined symmetrical elution.

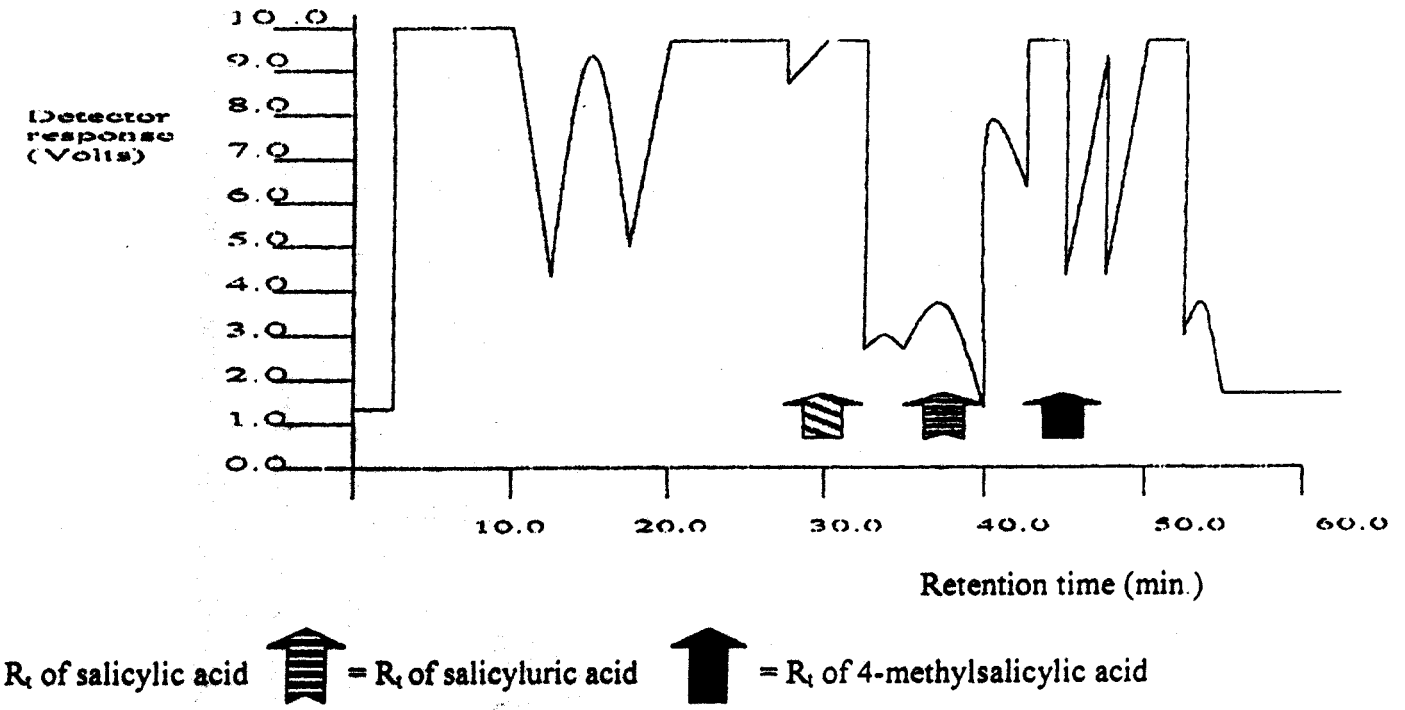
As a starting point, and in the absence of reliable information concerning salicylates in urine of people not taking salicylate drugs, it was decided to examine the method that had been developed by Blacklock et al [88] to determine SA in serum to see if it might be suitable. Acidified urine was extracted with ethyl acetate to remove acidic and neutral hydrophobic compounds, and these were chromatographed according to the method of Blacklock et al [88]. Although

oxidisable compounds with R_ts close to those of SA, SU and the internal standard (4-methylsalicylic acid) were eluted, the extract contained large amounts of interfering substances (see Fig.6), and it was concluded that the method was unsuitable for the determination of SA and SU in urine.

Knowing that the C18 column used in this work should retard elution of less polar compounds, and that elution of these hydrophobic materials should be accelerated by mobile phases of decreased polarity, it was decided to investigate the effects produced by mixing two mobile phases containing vastly different concentrations of methanol. The ternary unit of the chromatographic system provides a means of mixing these phases so that the content of methanol in the eluent might be altered in a flexible and controlled manner. Mobile Phase C (100% citrate buffer) and Mobile Phase A (50% citrate buffer: 50% methanol v/v) were selected.

Solutions containing known concentrations of SA and SU and an extract of urine (containing the internal standard) were prepared (see section 2.3.4.) After application to the chromatographic column and elution with mobile phase C, SA, SU and 4-methylsalicylic acid, as might be expected, had R_ts in excess of 60min. When elution was with mobile phase A the compounds were eluted very rapidly (4.6, 10.3 and 8.1 min respectively) and many other compounds in the extract of urine had R_t close to those of SA, SU and 4-methylsalicylic acid. These results indicate clearly that, in order to achieve a satisfactory resolution of the prospective determinands from many other materials extracted from urine, it would be

Figure 6. Chromatography of an extract of urine employing the extraction procedure and chromatographic conditions of Blacklock et al [88]

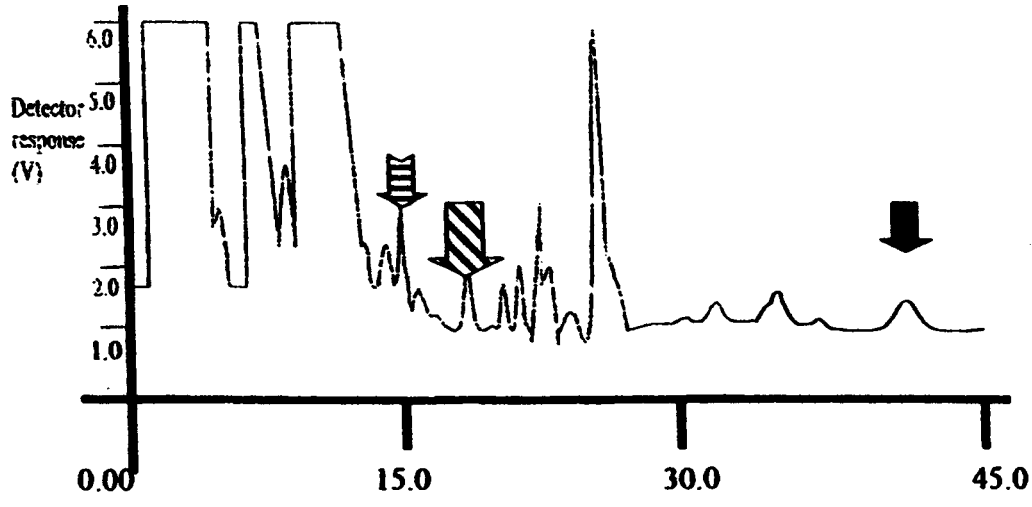


Internal standard was added to urine (0.5ml) of a person who had not taken salicylate drugs and the mixture was acidified and extracted with ethyl acetate as described in section 2.3.4. A portion (50µl) of the reconstituted extract was applied to the column and elution was according to the programme of stepwise elution described by Blacklock et al [88].

necessary to develop a programme of gradient elution. The strategy adopted was to try to retain SA, SU and 4-methylsalicylic acid on the column and to elute as many of the interferents as possible. The separation of SA, SU and 4-methylsalicylic acid then might be achieved by gradually decreasing the polarity of the eluent. A straightforward way of examining this idea was to use initially mobile phase C (citrate buffer) as eluent so that the three compounds of interest would be strongly retarded, and then to decrease the polarity of the eluent in a single step by eluting with mobile phase A (50% methanol: 50 % citrate buffer v/v). The final step would be to re-equilibrate the column using mobile phase C prior to application of the next extract. However, when this tactic was employed to try to separate the compounds, SA and 4-methylsalicylic acid were resolved while SU was eluted at approximately the same time as an unknown interferrent.

Guided by this observation, it was decided to try to achieve the clean separation of SU by decreasing the polarity of the eluent used initially. This was done by starting elution with a mixture containing 50% mobile phase A and 50% mobile phase C and then gradually increasing the polarity of the eluent in a series of smaller steps (see the legend of Fig. 7). However, the difference in the R_ss of SU and those of the substances eluted immediately before and after it were very small. The relevant resolution factors were less than unity (0.66 and 0.16, respectively) [see Fig. 7].

Figure 7. Chromatography of an extract of urine using a scheme of step-wise elution



= R_t of salicyluric acid

= R_t of salicylic acid

= R_t of 4-methylsalicylic acid

Urine (0.5ml) of a person who had not taken salicylate drugs (to which had been added internal standard and HCl) was extracted with ethyl acetate as described in section 2.3.4. A portion (50µl) of the reconstituted extract was applied to the column and elution at 0.5 ml per min was according to the step-wise programme as detailed below. Mobile phase A (50% citrate buffer: 50% methanol v/v, pH 4.0). Mobile phase C (100% citrate buffer, pH 3.8).

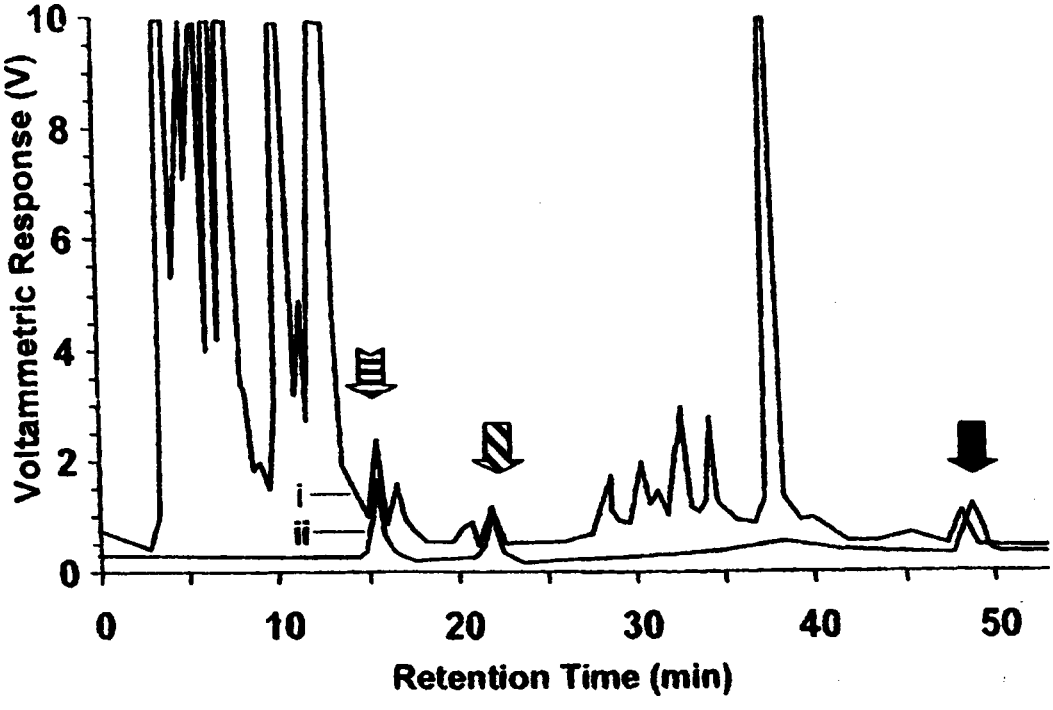
	Composition of eluent (v/v)	
Time of elution (min.)	% mobile phase A	% of mobile phase C
00.00 – 02.00	50	50
02.10 – 03.00	40	60
03.10 – 04.00	30	70
04.10 – 05.00	20	80
05.10 – 06.00	10	90
06.10 – 10.00	5	95
10.00 – 15.00	0	100
15.10 – 45.00	100	0
45.00 – 59.00	50	50

Satisfactory separation of the three compounds of interest had almost been achieved. The chromatographic instrumentation allows changes to be made in the rate of flow of eluent. The programme of elution described in Fig. 7 was modified so as to decrease the rate of elution (from 0.5 ml per min to 0.3 ml per min) just prior to the R_t of SU and this resulted in a clean separation of SU from the compounds eluted immediately before and immediately after it. This alteration of rate of flow allowed the clean separations of SA and SU but it caused a deterioration in the separation of 4-methylsalicylic acid. However, a further reduction in the rate of flow from 0.5ml per min to 0.3 ml per min just prior to the R_t of 4-methylsalicylic acid restored the clean separation of the internal standard. Alteration of the flow rate at these two points within the programme of elution resulted in the resolution of SA, SU and 4-methylsalicylic acid from each other and from the other neutral and acidic hydrophobic compounds extracted from urine (see Fig. 8). Table II shows the details of this successful programme of stepwise gradient elution.

3.1.2 The identification of SA and SU in urine.

The chromatographic method described in Table II allowed the complete separation of the compounds in urine that had R_ts very close to those of SA and SU. Now it was necessary to establish the identities of these two compounds. Identification used three different approaches. The first approach was to alter the chromatographic conditions to see if the chromatographic characteristics of the compounds suspected of being SA and SU were altered in the same way as those of the authentic substances. The second approach was to utilize the enzyme

Figure 8. Fractionation of the hydrophobic compounds of urine by HPLC: separation of salicylic acid, salicyluric acid and 4-methylsalicylic acid



(i) Extract of urine (see legend of Fig. 7)

(ii) Mixture of salicylic acid, salicyluric acid and 4-methylsalicylic acid

= R_t of salicylic acid (resolution factors 1.51,1.87)

= R_t of salicyluric acid (resolution factors 1.51,3.64)

= R_t of 4-methylsalicylic acid (resolution factors 1.54, 3.33)

Elution was according to the programme of stepwise elution described in Table II.

salicylate hydroxylase to see if the two unknown compounds were transformed. Salicylate hydroxylase catalyses the conversion of *o*-hydroxybenzoates into the respective catechols. Finally, the two unknown compounds were separated by HPLC, extracted from the eluates, esterified and examined by using GCMS.

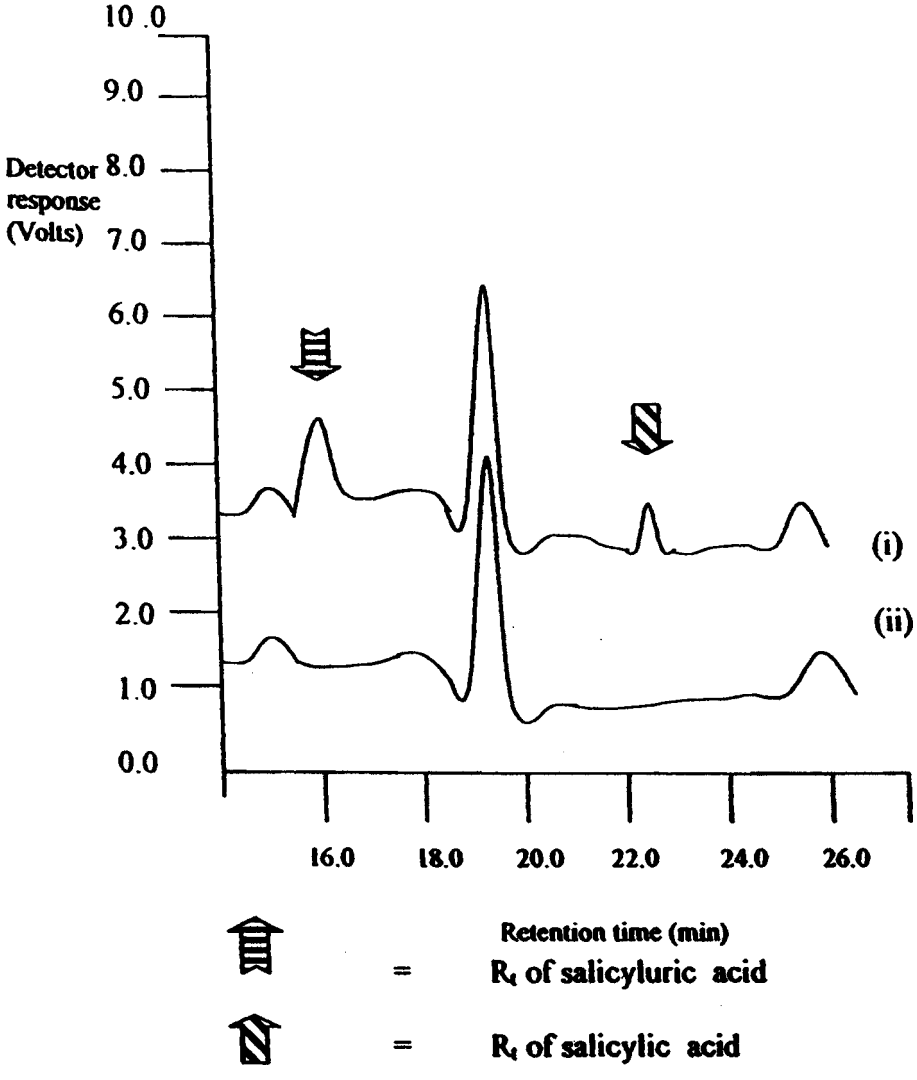
Two of the hydrophobic compounds extracted from the urine of people who had not taken salicylate drugs had R_ts that were very similar to those of authentic SA (21.7 min) and SU (15.8 min) [see Fig.8]. When the conditions of elution detailed in Table II were changed (by increasing the concentration of methanol in mobile phase A by 15% from 50% methanol: 50% citrate buffer (v/v) to 65% methanol: 35% citrate buffer (v/v) the R_t of authentic SA and SU were decreased to 16.8 min and 10.2 min respectively. Those of two of the hydrophobic compounds extracted from urine were decreased also to values close to these (17.0 min and 10.7 min respectively).

The identities of the unknown compounds in urine suspected of being SA and SU were then investigated using salicylate hydroxylase. When aqueous solutions of SA and SU (concentration range, 0.2 – 1.0 μmol/l) were treated with the enzyme, the phenolic acids, at all of the concentrations used, were transformed completely within 15 minutes; they were not detected after chromatography of extracts of the reaction mixtures using the conditions of elution described in Table II. When urine was treated with the enzyme the compound suspected of being SA was transformed completely and about 75% of the compound suspected of being SU was transformed. However, when urine was treated with salicylate hydroxylase for 30

minutes the compound suspected of being SU was transformed completely; it could not be detected in extracts of the reaction mixture (see Fig. 9). When salicylate hydroxylase was omitted from reaction mixtures containing either urine or SA or SU, the unknown compounds were not transformed and they were eluted from the column with R_ts identical to those of SA and SU.

The hydrophobic compounds extracted from urine with ethyl acetate and suspected to be SA and SU were isolated by HPLC using the chromatographic conditions detailed in Table II (see section 2.3.5). The fractions containing the compounds were acidified and extracted with ethyl acetate. After evaporation of the organic solvent the dried extracts were treated with acetyl chloride in methanol and the mixtures were dried with a stream of N_2 (see section 2.3.7). The residues were redissolved in ethyl acetate and when these solutions were applied to the gas chromatographic column it was found that they contained compounds with R_ts very similar to those of methyl salicylate and methyl salicylurate. These compounds had mass spectra that were very similar to those of methyl salicylate and methyl salicylurate (see Table IV and Fig. 10). The mass spectra of the unknown compounds resembled very closely those of methyl salicylate and methyl salicylurate catalogued in the Pfleger, Mauer and Weber library of mass spectra [92]. From all of this evidence it was concluded that SA and SU were components of the urine of people who had not taken salicylate drugs.

Figure 9. Chromatography of (i) an extract of urine and (ii) an extract after treatment of urine with salicylate hydroxylase



Urine (0.5 ml) of a person who had not taken salicylate drugs was acidified and extracted with ethyl acetate as described in section 2.3.4. A second portion of urine (0.5ml) was treated with salicylate hydroxylase for 30 min as described in section 2.3.7, acidified and extracted with ethyl acetate as described in section 2.3.4. Portions (50µl) of the reconstituted extracts were applied to the column and elution was according to the stepwise programme detailed in Table II.

Figure 10. Principal ion mass spectra of compounds extracted from urine, separated by HPLC and treated with acetyl chloride in methanol. (a) salicylic acid treated with acetyl chloride in methanol, (b) an unknown compound with the HPLC R_t of salicylic acid treated with acetyl chloride in methanol (c) salicyluric acid treated with acetyl chloride in methanol (d) an unknown compound with the HPLC R_t of salicyluric acid treated with acetyl chloride in methanol.

Figure 10a.
Relative abundance

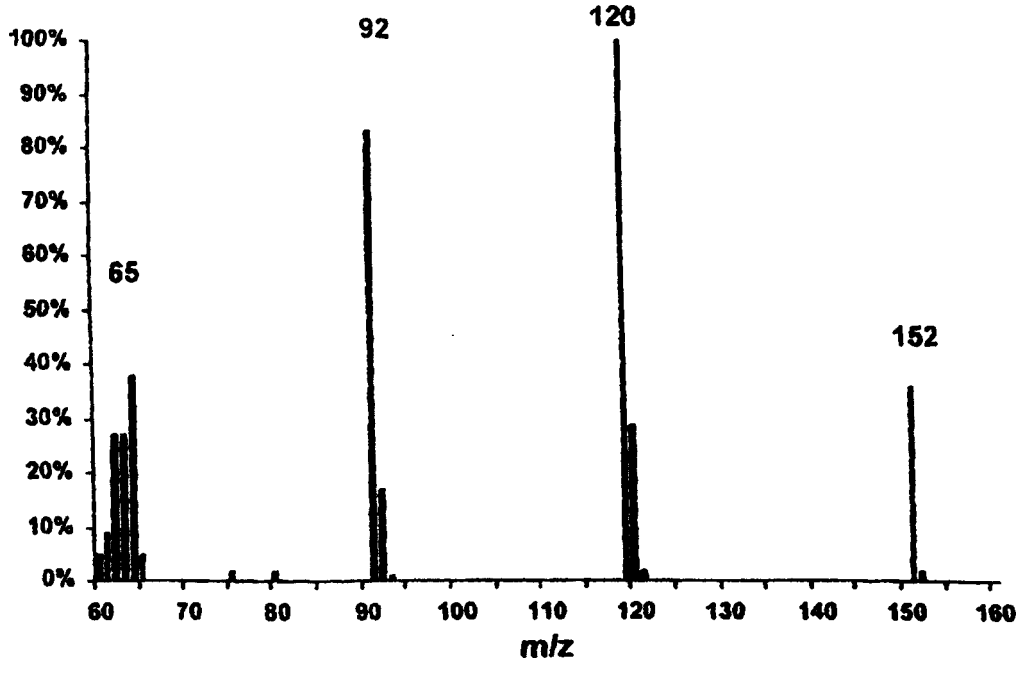


Figure 10b
Relative abundance

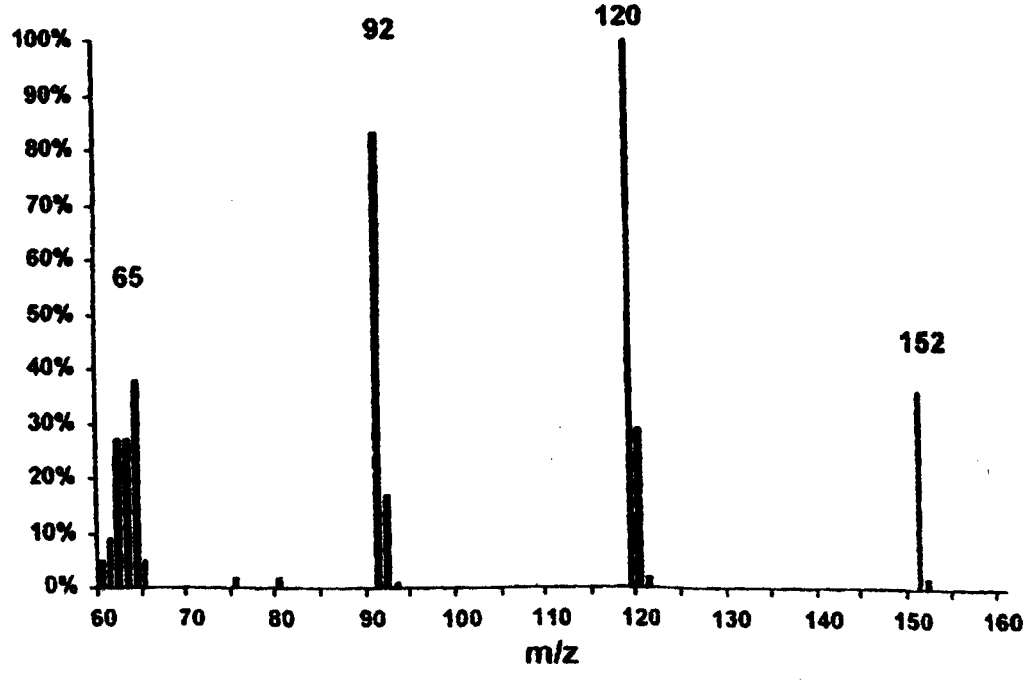


Figure10c.

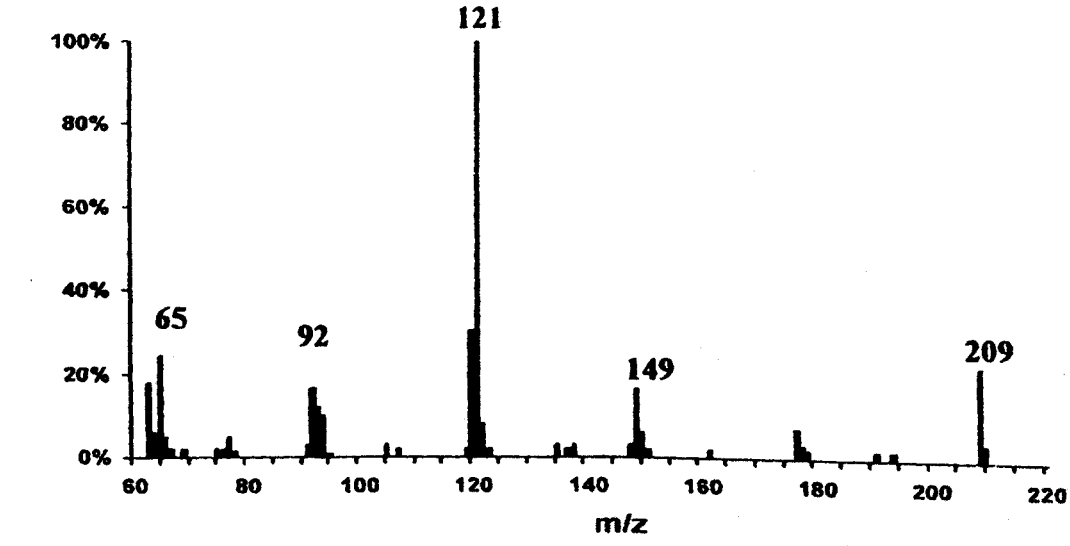


Figure10d.

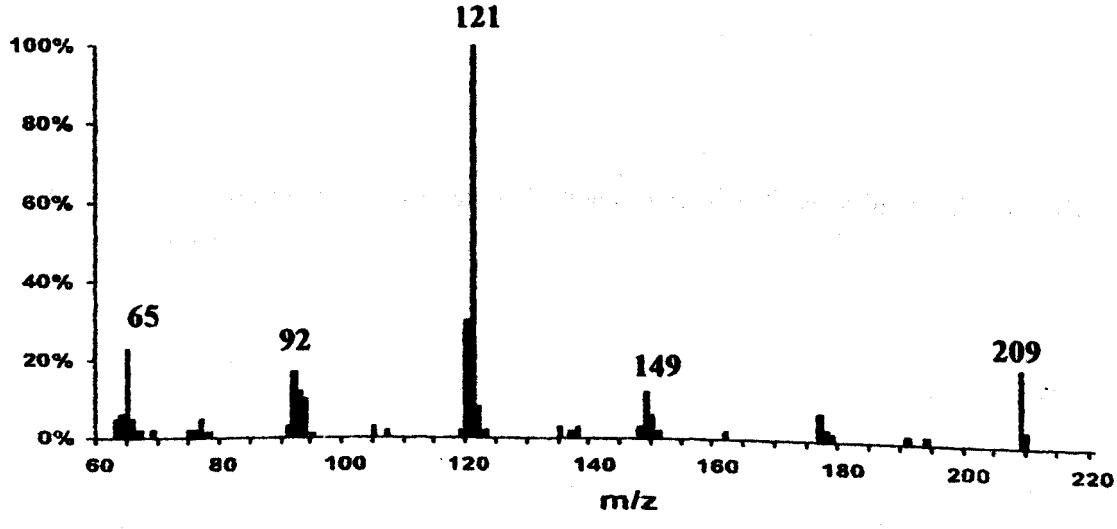


Table IV - GCMS of salicylic acid (SA) and salicyluric acid (SU) treated with acetyl chloride in methanol, methyl salicylate and the compounds isolated from extracts of urine by HPLC and treated with acetyl chloride in methanol

Compound	R_t (min)	Principal ions in spectrum: Mass	Relative abundance
Methyl salicylate	7.35	65	40
		92	82
		120	100
		* 152	40
SA treated with acetyl chloride in methanol	7.35	65	39
		92	82
		120	100
		* 152	36
Substance extracted from urine and treated with acetyl chloride in methanol	7.48	65	38
		92	83
		120	100
		* 152	36
Substance extracted from urine and treated with acetyl chloride in methanol	14.96	65	23
		92	16
		121	100
		149	12
		* 209	18
SU treated with acetyl chloride in methanol	14. 83	65	24
		92	18
		121	100
		149	13
		* 209	20

See also Fig. 10.
* Molecular ion
The molecular masses of methyl salicylate and methyl salicylurate are 152.1 and 209.2 respectively.

3.1.3 Quantification of SA and SU in urine.

(a) Calibration

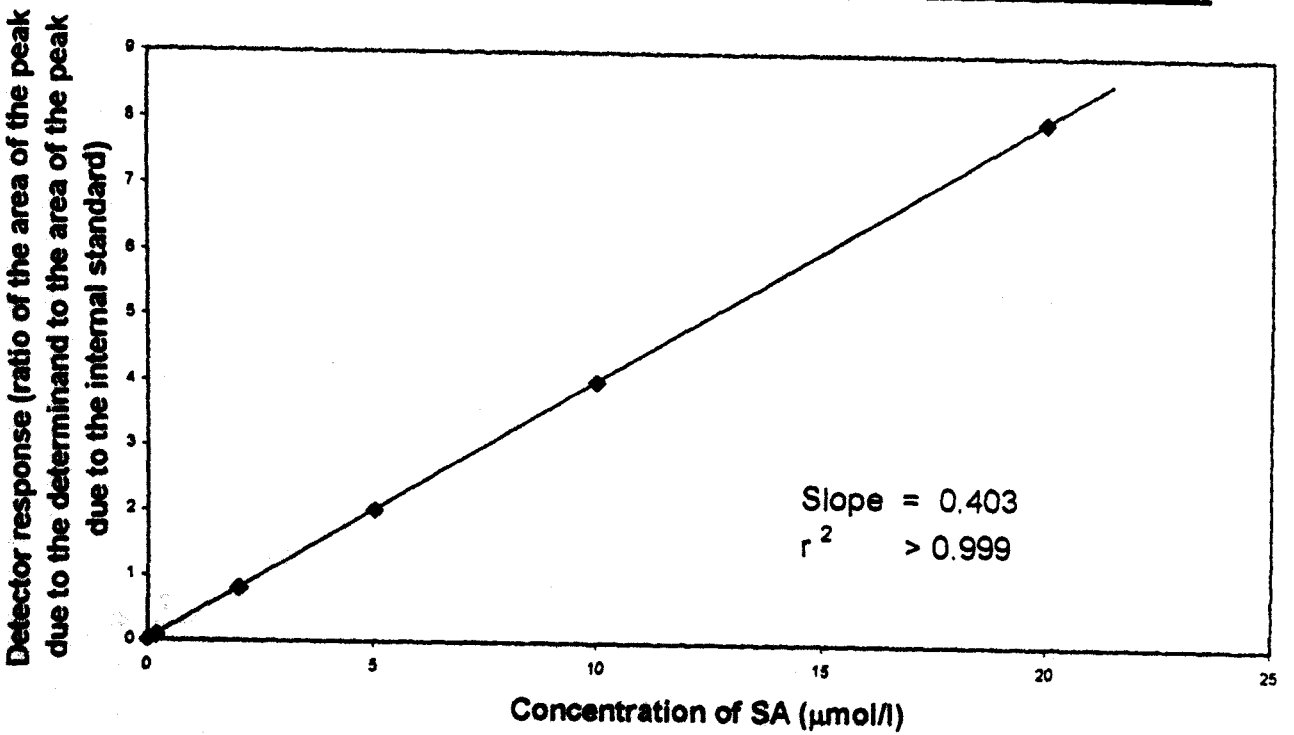
Having established that SA and SU were components of the urine of people who have not taken salicylate drugs, the possibility of using the electrochemical (oxidative) method of their detection in a reliable, quantitative fashion was examined.

Portions (50µl) of solutions of SA and SU in water in the concentration ranges 0.02 – 20.0 µmol/l and 0.2 – 200.0 µmol/l respectively were chromatographed employing the conditions described in sections 2.3.5 and 2.3.6. Graphs of the ratio of the area of the peak due to the determinand to the area of the peak due to the internal standard (4-methylsalicylic acid) against concentration of the determinand were constructed (see Figs 11,12). Fits were linear and passed through the origin with $r^2>0.998$.

(b) Efficiency of extraction

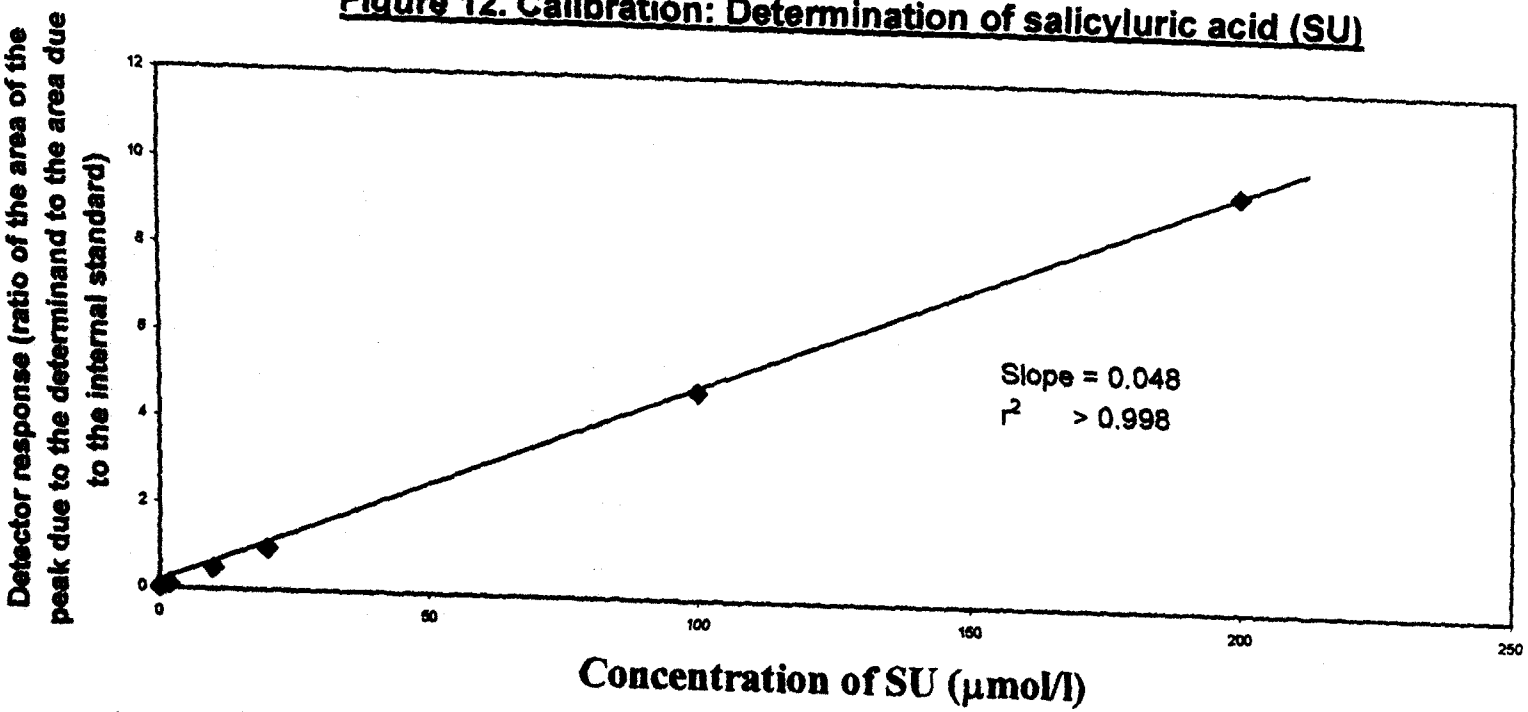
The efficiency of removal of the compounds from acidified urine into ethyl acetate was assessed by comparing the concentrations of SA and SU found in urine (0.837 µmol/l and 5.564 µmol/l respectively) with the concentrations determined after known concentrations (0.2 and 2.0 µmol/l respectively) of the determinands had been added to the urine. It was observed that 85% of the SA and 87% of the SU that had been added to the urine were extracted. These values indicate that the method of extraction is acceptably efficient.

Figure 11. Calibration: Determination of salicylic acid (SA)



The best fit was calculated using the method of least squares assuming that the variances of values on the ordinate axis were identical.

Figure 12. Calibration: Determination of salicyluric acid (SU)



The best fit was calculated using the method of least squares assuming that the variances of values on the ordinate axis were identical.

(c) Reliability

Extraction of acidified urine with ethyl acetate followed by separation of SA and SU by HPLC with electrochemical quantification appear to form the basis of a method to determine their concentrations in urine. To ensure that this type of method is not only accurate and sensitive but also reliable, the repeatability (intra-assay precision) and the reproducibility of this method (inter-assay precision) were determined as described in section 2.3.8. The results obtained (Table V) showed that the method can be used with confidence for determining the concentrations of SA and SU in urine.

(d) The limit of detection of the method

Having developed a reproducible and reliable method for determining the concentrations of SA and SU in urine it was necessary also to address the question of its sensitivity. The limit of detection of the method (as described in section 2.3.8.) for both SA and SU in urine was 0.005μmol/l. During the course of this work the lowest concentrations of SA and SU in urine determined by using this method were 0.014 μmol/l and 0.011 μmol/l respectively. In four samples of urine the concentrations of SA and SU were lower than the limit of detection.

(e) The stability of SA and SU in urine

The reason for developing a method to determine the concentrations of SA and SU in urine was to allow the assessment of the extent of exposure of people to salicylates. However, these assessments would be valid only if the determinands were sufficiently stable in urine. The concentrations of SA and SU in freshly

Table V - The inter-assay and intra-assay precision of the method for determining the concentration of salicylic acid and salicyluric acid in urine

	Coefficient of variation. Salicylic acid * (mean concentration 0.839 μmol/l)	Coefficient of variation. Salicyluric acid * (mean concentration 5.564 μmol/l)
Intra-assay precision	0.03%	0.07%
Inter-assay precision	2.48%	2.43%

See the text and section 2.3.8

* The mean concentration of SA and SU observed in the urine that were used to examine precision occur at the high ends of the ranges of concentration of these compounds found in urine from people who had not taken salicylate drugs (see the data of Table VII).

collected urine were determined. These concentrations were determined again after the urine had been stored for 24h at room temperature, at 4°C and at -20°C. From the values recorded in Table VI it was concluded that both SA and SU did not decompose significantly under the conditions of collection and storage employed.

3.2 SA and SU in the urine of people with different diets

Now that a sensitive, accurate and reliable method to determine SA and SU in urine had been developed, their concentrations in the urine (collected over 24h time periods) of various groups of people were determined. The amounts of SA and SU excreted should provide information concerning the extent of exposure of these people to salicylates.

Blacklock et al [88] reported that higher concentrations of SA were present in the serum of vegetarians and in the serum of people taking low-dose aspirin than in the serum of people who had not taken aspirin and who had an unrestricted diet. Therefore, it was decided to determine the concentrations of SA and SU in urine from these four groups of people. One of the groups had a strict vegetarian diet; a second group had an unrestricted diet. Neither of these two groups of people had taken salicylate drugs. The other two groups of people had unrestricted diets and had taken aspirin at doses of either 75mg per day or 150 mg per day for more than two months. Table VII shows the concentrations in urine and the amounts of SA and SU excreted by these four groups of people in a period of 24h. The amounts of SU

Table VI - The stability of salicylic acid and salicyluric acid in urine

Temperature /Time of storage	Concentration of salicylic acid (μmol/l)	Concentration of salicyluric acid (μmol/l)
Freshly collected	0.227	1.284
Room temp./24h	0.223	1.276
4°C /24h	0.219	1.345
-20°C /24h	0.234	1.312

See the text and section 2.3.8.

Table VII - The urinary concentrations and amounts of salicylic acid (SA) and salicyluric acid (SU) excreted by four groups of people

Group	Median concentration of SA (μmol/l)	Median concentration of SU (μmol/l)	Median amount of SA excreted (μmol/24h)	Median amount of SU excreted (μmol/24h)
1	0.21 (range, 0.014 – 1.12)	1.73 (range, 0.537 – 9.72)	0.31 (range, 0.01 – 2.01)	3.91 (range, 0.87 – 12.23)
2	0.55 (range, 0.014 – 2.61)	7.25 (range, 2.82 – 22.66)	1.19 (range, 0.02 – 3.55)	11.01 (range, 4.98 – 26.60)
3	** 0.22 (range, <0.005 – 2.66)	100.41 (range, 13.99– 254.85)	** 0.41 (range, <LOD – 3.88)	170.69 (range, 13.15 – 377.18)
4	0.15 (range, 0.025 – 65.23)	* 68.62 (range <0.005– 220.21)	0.36 (range, 0.04 – 7.10)	* 165.17 (range, <LOD - 429.12)

Group 1 (n = 27): people not taking salicylate drugs.

Group 2 (n = 21): strict vegetarians not taking salicylate drugs.

Group 3 (n = 15): people taking 75 mg of aspirin per day
(** including one individual whose urine contained a concentration of SA which was less than the limit of detection).

Group 4 (n = 25): people taking 150 mg of aspirin per day
(*including one individual whose urine contained a concentration of SU which was less than the limit of detection).

Groups 1,3 and 4 had unrestricted diets

excreted by the group of vegetarians and the two groups of people who had been taking aspirin were significantly higher than the amount excreted by the group of non-vegetarians ($p < 0.0001$, in all three Mann-Whitney U-tests). The amount of SA excreted by the group of vegetarians was significantly higher than the amount excreted by the group of non-vegetarians ($p = 0.0166$, Mann-Whitney U-test).

3.3. The concentration of SA in the blood of people from Southern India.

By using the method of Blacklock et al [88] the concentrations of SA in sera from a group of people ($n = 21$) who live in a rural part of southern India and who had not taken salicylate drugs were determined. These people had a diet that was known to contain large quantities of fruit, vegetables and cereals cooked with substantial quantities of herbs and spices. The range observed was 0.050 – 0.635 μmol/l, and the median concentration of SA was 0.263 μmol/l which is more than double the median concentration found in sera from the group of vegetarians studied by Blacklock et al [88] (see Table VIII).

The concentrations of SA observed in the blood of Southern Indians were significantly higher than those observed for the vegetarians and the non-vegetarians ($p < 0.001$, from both Mann-Whitney U tests). However, they were significantly lower than the values observed [88] for the group of people who had been taking 75 mg of aspirin per day ($p < 0.001$, Mann-Whitney U test).

3.4. The determination of SA in foodstuffs

The incidence of colorectal cancer in India is low and it appears that it increases

Table VIII - The concentration of salicylic acid in the blood of various groups of people

	Median concentration of salicylic acid (μmol/l)
Southern Indians (n = 21)	0.263 (range, 0.05 – 0.64)
Vegetarians (n = 37)	0.110 (range, 0.04 – 2.47)
Non-vegetarians (n = 39)	0.070 (range, 0.02 – 0.20)
Aspirin takers (75 mg/day) (n = 14)	10.03 (range, 0.23 – 25.40)

The values observed for vegetarians, non-vegetarians and the people receiving aspirin were determined by Blacklock et al [88].

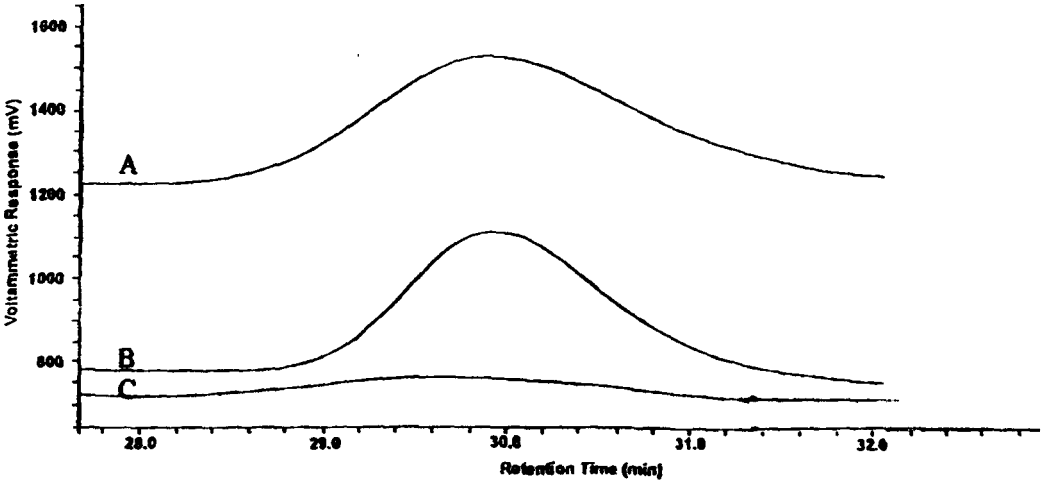
when members of its population migrate to other parts of the world. These observations suggest that there is a significant environmental component involved in the incidence of the disease. The incidence of colorectal cancer among people exposed to SA by consuming aspirin over long periods of time also is reported to be lower (by 40% - 50%) than the incidence reported in people who are not exposed to the phenolic acid to this extent. This information, in conjunction with the findings of higher concentrations of SA in sera of people who are vegetarians and the even higher concentrations in sera from the group of people living in rural India, prompted an investigation of the content of SA in various foodstuffs.

3.4.1. The identification of SA in foodstuffs

When an extract of soup was chromatographed according to the method of Blacklock et al [88], a substance with a very similar R_t to that of SA was eluted. Evidence as to the identity of this substance was obtained by altering the chromatographic conditions (by using isocratic elution with mobile phase A). Using these conditions the compound suspected to be SA was eluted at a R_t of 7.35 min. Under these conditions the R_t of SA was 7.36 min.

When the compounds extracted with ethyl acetate from portions of 4 homogenized soups were reconstituted in phosphate buffer and treated with salicylate hydroxylase (see section 2.3.7.) and chromatographed, the compound suspected of being SA was transformed (for typical results see Fig 13).

Figure 13. Transformation (catalysed by salicylate hydroxylase) of a compound present in soup



Chromatograms of:

A Compounds extracted from soup with ethyl acetate

B An aqueous solution of salicylic acid.

C Compounds extracted from soup treated with salicylate hydroxylase.

Portions of soup (1g) were acidified and extracted with ethyl acetate as described in section 2.3.4. The compounds extracted with ethyl acetate from one portion of soup were treated with salicylate hydroxylase (see section 2.3.7) The compounds extracted with ethyl acetate from a second portion of the soup were incubated in the absence of salicylate hydroxylase (see section 2.3.7.). The reaction mixtures were then re-extracted with ethyl acetate (see section 2.3.4), and brought to dryness. The residues were reconstituted in mobile phase B, and 50 µl of each solution were applied to the column. Elution was according to the programme of described by Blacklock et al [88].

The identity of the compound extracted from a soup (Co-op Tomato and Lentil) and from three spices (cumin, turmeric and paprika, obtained from a supplier of the Indian and Pakistani communities) was established by using GCMS. It was found that the hydrophobic compound extracted from the soups and spices and isolated by using HPLC (see section 2.3.7) after treatment with acetyl chloride had a gas chromatographic R_t and a mass spectrum that were very similar to those of SA after treatment with acetyl chloride in methanol (see Figs. 14 – 17 and Tables IX – XIII for typical spectra, relative abundances and values of R_t).

Table IX - GCMS of salicylic acid treated with acetyl chloride in methanol and a compound extracted from soup, isolated by HPLC, and treated with acetyl chloride in methanol

Compound	R_t (min)	Principal ions in spectrum	
		Mass	Relative abundance
Salicylic acid	7.35	65	39
		92	82
		120	100
		* 152	36
Substance extracted from soup	7.48	65	37
		92	81
		120	100
		* 152	35

See also Fig. 14.
* Molecular ion
The molecular mass of methyl salicylate is 152.1.

Figure 14. Principal ion mass spectra of (a) salicylic acid treated with acetyl chloride in methanol, (b) a compound extracted from soup (Co-op Tomato and Lentil), isolated by HPLC and treated with acetyl chloride in methanol.

Figure 14a.

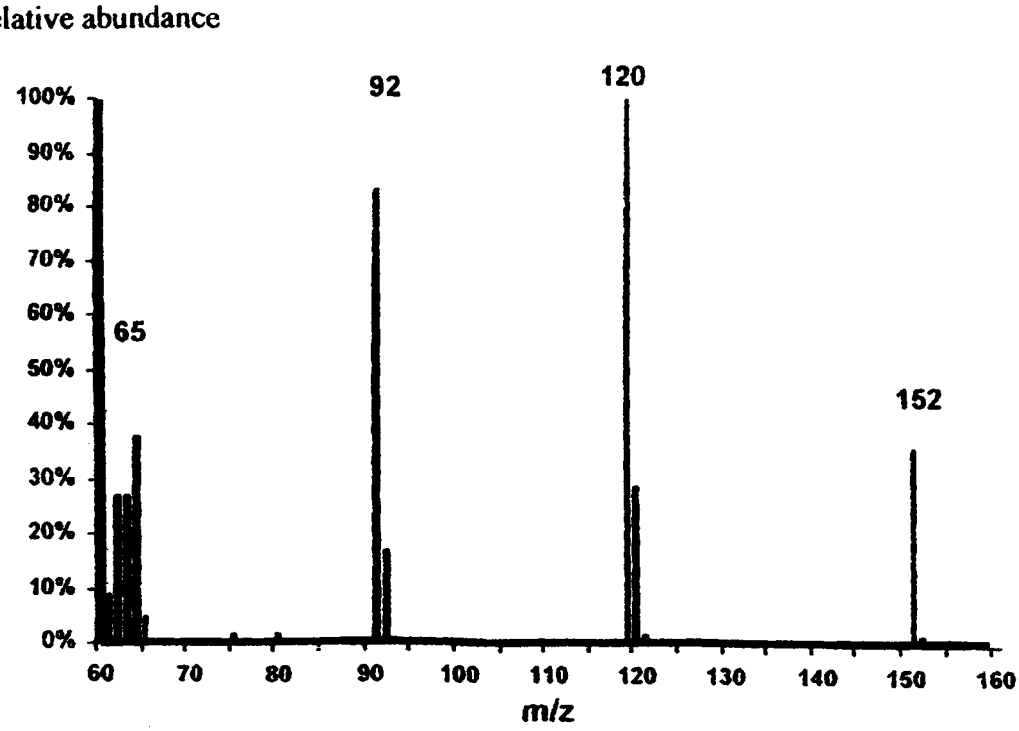


Figure 14b.

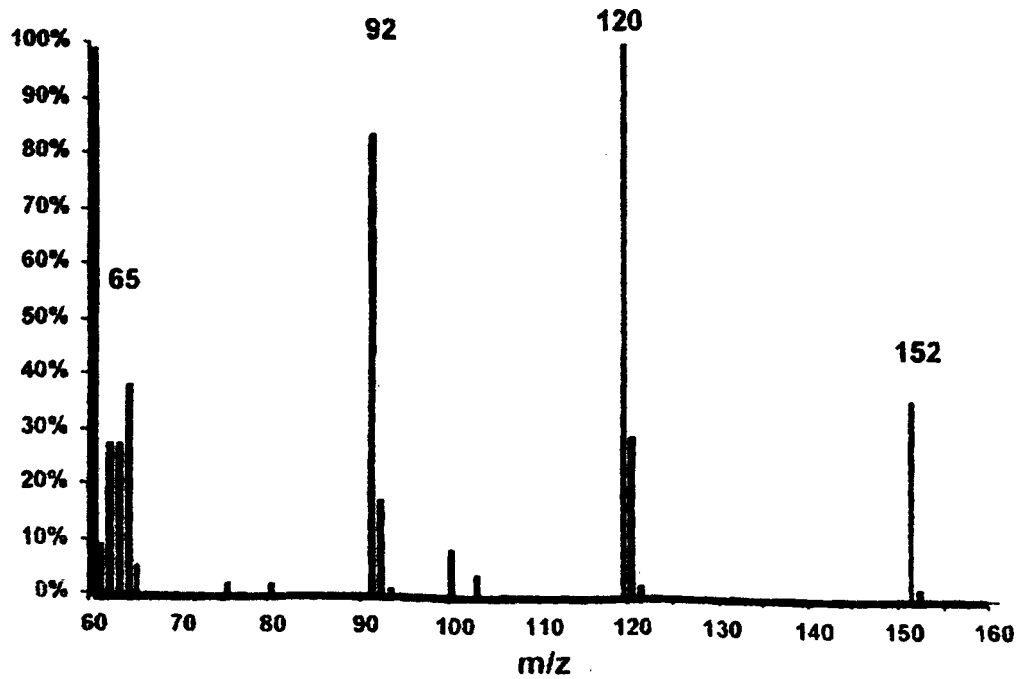


Table X - GCMS of salicylic acid treated with acetyl chloride in methanol and a compound extracted from cumin, isolated by HPLC, and treated with acetyl chloride in methanol

Compound	R_t (min)	Principal ions in spectrum: Mass	Relative abundance
Salicylic acid	7.35	65	39
		92	82
		120	100
		*152	36
Substance extracted from cumin	7.45	65	36
		92	82
		120	100
		*152	37

See also Fig. 15.
* Molecular ion
The molecular mass of methyl salicylate is 152.1.

Figure 15. Principal ion mass spectra of (a) salicylic acid treated with acetyl chloride in methanol, (b) a compound extracted from cumin, isolated by HPLC and treated with acetyl chloride in methanol.

Figure 15a.

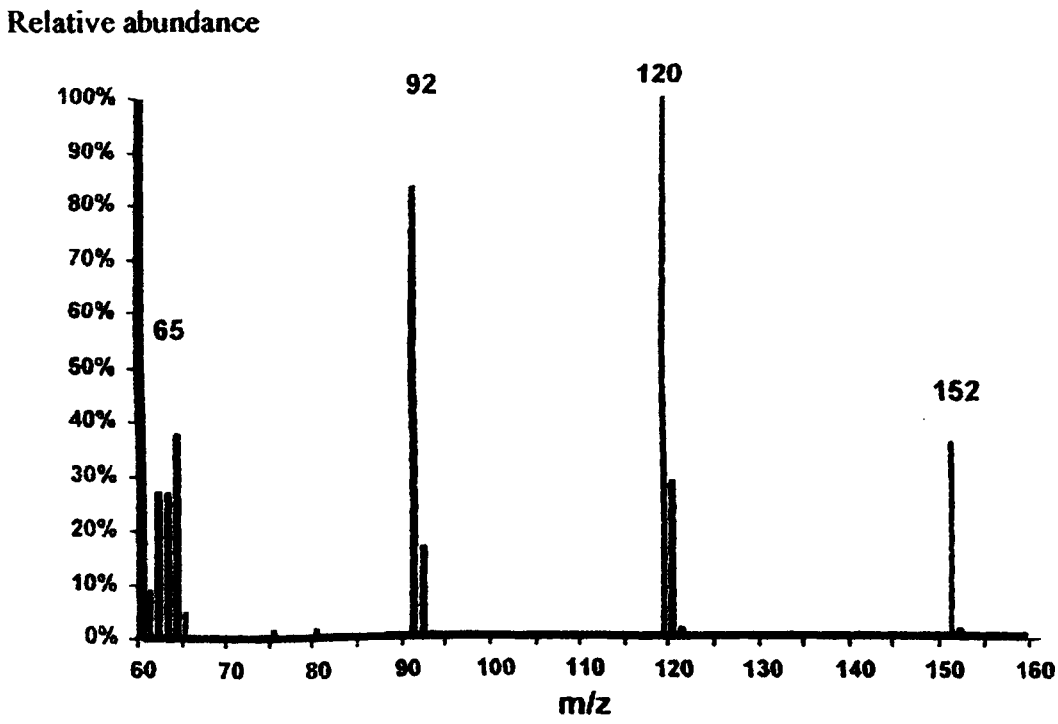


Figure 15b.

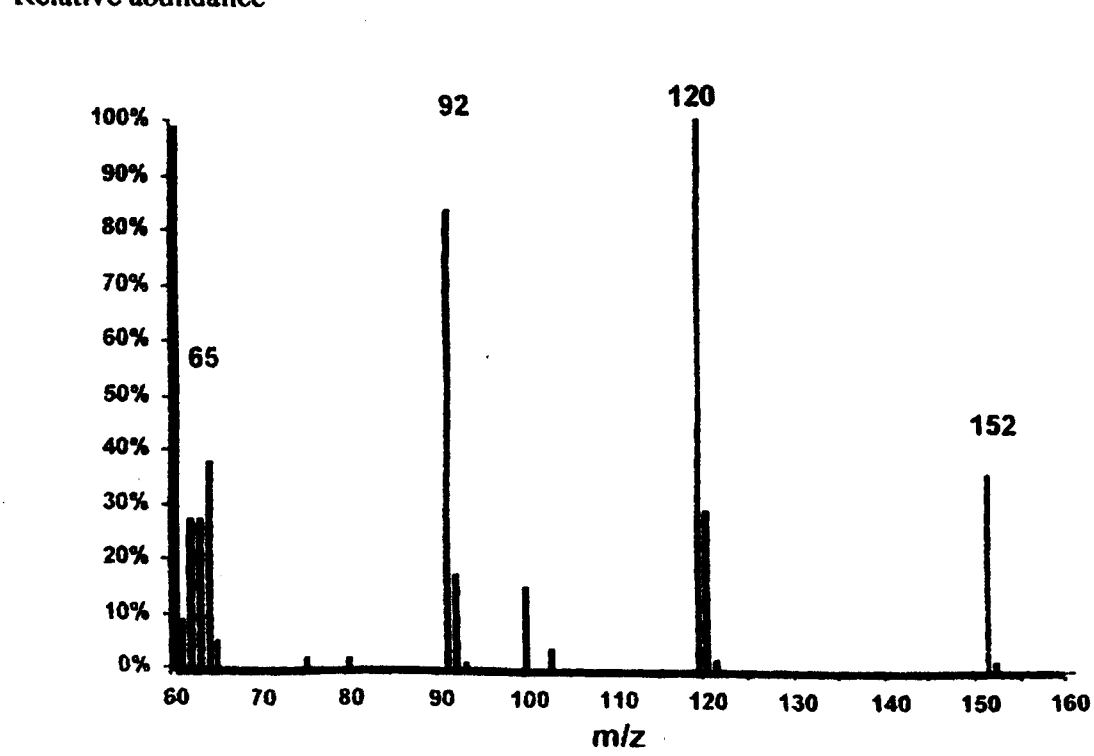


Table XI - GCMS of salicylic acid treated with acetyl chloride in methanol and a compound extracted from paprika, isolated by HPLC, and treated with acetyl chloride in methanol

Compound	R_t (min)	Principal ions in spectrum	
		Mass	Relative abundance
Salicylic acid	7.35	65	39
		92	82
		120	100
		*152	36
Substance extracted from paprika	7.48	65	36
		92	82
		120	100
		*152	35

See also Fig. 16.
* Molecular ion
The molecular mass of methyl salicylate is 152.1.

Figure 16. Principal ion mass spectra of (a) salicylic acid treated with acetyl chloride in methanol, (b) a compound extracted from paprika, isolated by HPLC and treated with acetyl chloride in methanol.

Figure 16a.

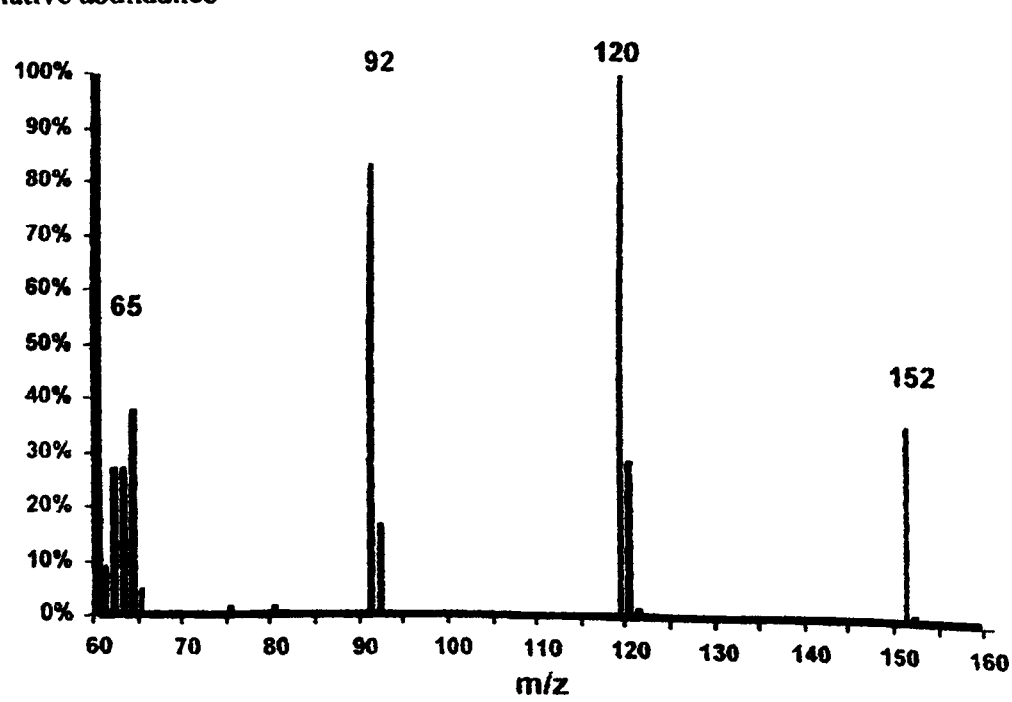


Figure 16b.

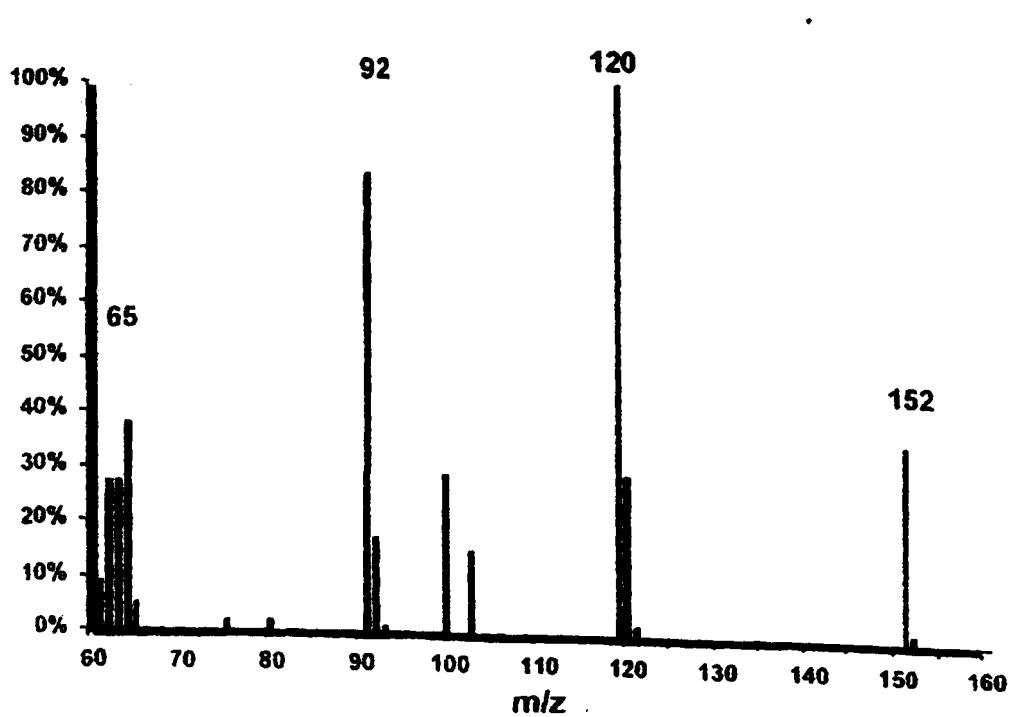


Table XII - GCMS of salicylic acid treated with acetyl chloride in methanol and a compound extracted from turmeric, isolated by HPLC, and treated with acetyl chloride in methanol

Compound	R_t (min)	Principal ions in spectrum	
		Mass	Relative abundance
Salicylic acid	7.35	65	39
		92	82
		120	100
		*152	36
Substance extracted from turmeric	7.45	65	38
		92	81
		120	100
		*152	36

See also Fig. 17.
* Molecular ion
The molecular mass of methyl salicylate is 152.1.

Figure 17. Principal ion mass spectra of (a) salicylic acid treated with acetyl chloride in methanol, (b) a compound extracted from turmeric, isolated by HPLC and treated with acetyl chloride in methanol.

Figure 17a.

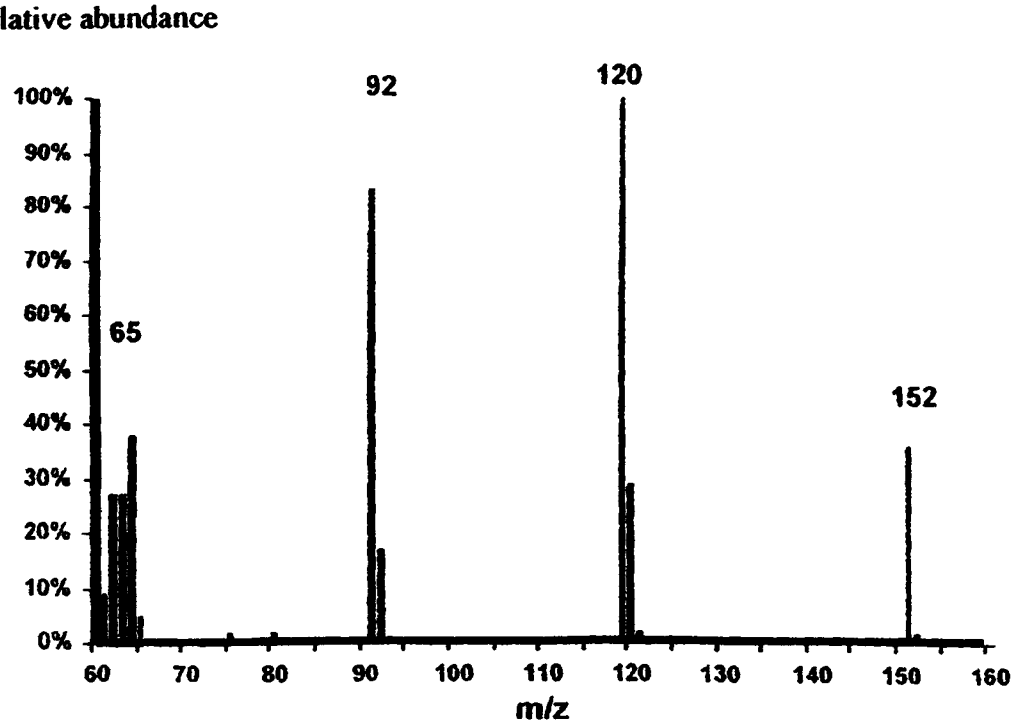
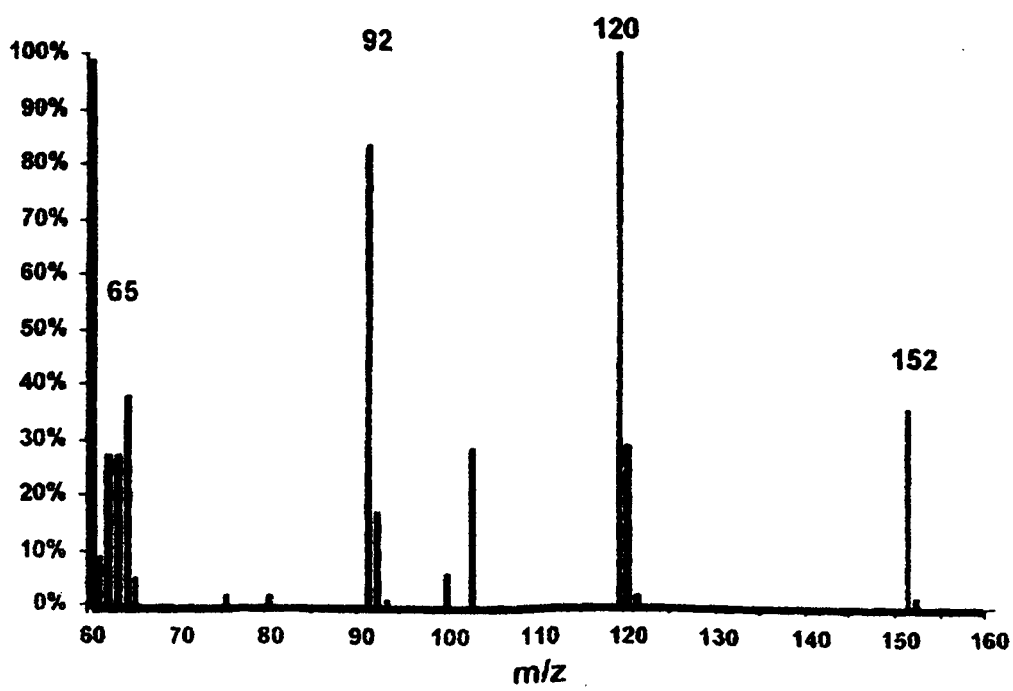


Figure 17b.



3.4.2. The quantification of SA in foodstuffs

The method used by Blacklock et al [88] to determine the concentration of SA in serum was examined to see if it might be suitable for the determination of SA in food. The acidic and neutral hydrophobic compounds were extracted with ethyl acetate from acidified portions of homogenized soups, homogenized cooked foods, powdered spices and from portions of wine. They were chromatographed using the programme of step-wise gradient elution described by Blacklock et al [88] (see section 2.3.5.). SA and 4-methylsalicylic acid (the internal standard) were separated cleanly (see for example Fig. 18).

Now that it had been established that SA is present in foodstuffs and that it could be separated efficiently, the reliability of the method of Blacklock et al [88] for the determination of the concentration of SA in food was assessed.

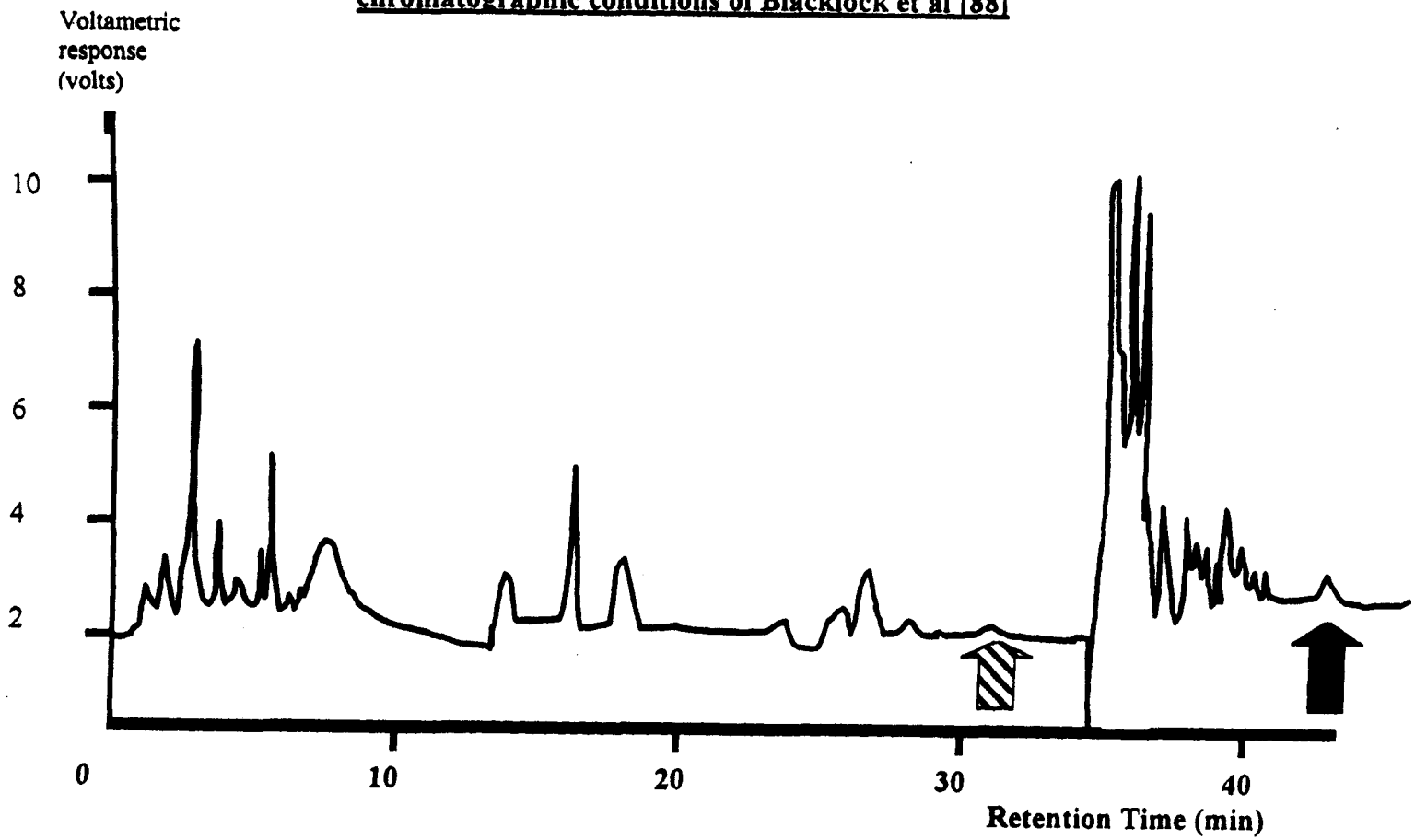
(a) The reliability of the method

The reproducibility of the method (inter-assay precision) was determined as described in section 2.3.8. using 4 different soups (see also the legend of Table XIII). The CV ranged between 0.7 and 6.8%.

The intra-assay precision of the method was evaluated as described in section 2.3.8 using values from 22 duplicate sets of determinations. The CV was 2.4%.

The method was found to be less precise than the method developed to determine the concentration of SA in urine and it is possible that the variability of the extraction of SA from the complex semi-solid matrix of soup might have

Figure 18. Chromatography of an extract of soup employing the extraction procedure and chromatographic conditions of Blacklock et al [88]



A portion of soup (0.5ml), to which HCl and internal standard had been added, was extracted with ethyl acetate as described in section 2.3.4. A portion (50μl) of the extract reconstituted in 0.5ml of mobile phase B was applied to the column and elution was according to the programme of stepwise elution described by Blacklock et al [88].

TABLE XIII - Inter-assay precision of the method used to determine the concentration of salicylic acid (SA) in foodstuffs

Soup	Mean concentration of salicylic acid (ng/g)	CV (%)
Tesco Red Pepper and Tomato	8 (n = 5)	1.2
Heinz Carrot and Coriander	15 (n = 4)	6.8
Home Made	41 (n = 5)	0.7
Asda Country Vegetable	83 (n = 4)	3.6

The concentrations of SA in four soups were determined by using the method of Blacklock et al [88] (see sections 2.3.3 – 2.3.5).

n = number of separate determinations.

contributed to the wider range of inter- assay precision (0.7 to 6.8%).

(b) The efficiency of the extraction step of the method

To assess the efficiency of the extraction stage of the method, paired portions of five different varieties of soup were examined (see section 2.3.4). Each pair included a portion to which 15ng of SA per 1g of soup had been added and a portion that had not received any additional SA. The amounts of SA present in extracts of the soups were determined and the efficiency of extraction into ethyl acetate was calculated. It was found that 78%, 72%, 83%, 77% and 84% of the SA added to the five different soups was recovered.

3.5. SA in the diet

SA is a defence hormone of plants, and plants grown organically are not protected by the application of pesticides and fungicides. Plants reared organically therefore, might contain more SA than plants grown by conventional means. The foodstuffs prepared from organically-grown ingredients might also contain greater amounts of SA.

To see if foods manufactured from organically-grown ingredients contained different amounts of SA from food manufactured from conventional ingredients, soups from a variety of manufacturers were examined and their concentrations of SA were determined.

3.5.1. The content of SA in soups.

The soups (n = 45) were homogenized and portions (1g) were acidified and

extracted with ethyl acetate (see section 2.3.4). The SA extracted was determined using the method of Blacklock et al [88] (see Table XIV). The content of SA in vegetable soups made using ingredients claimed to have been grown organically (n = 11) were compared with those in soups made using ingredients produced conventionally (n = 24). The median amount of SA in the organic soups [117.0 ng/g (range, 8 – 1040ng/g)] was significantly higher than that of the conventional soups [20.0 ng/g (range, 0 – 248 ng/g)] (p = 0.0032, Mann-Whitney U-test).

3.5.2 The content of SA in wines.

As an additional test of the hypothesis that food produced from organically-grown plants might contain more SA than plants produced from food grown conventionally, the concentrations of SA in various wines were determined.

This selection of wines included red and white organic and conventional wines, as well as a late-harvested wine (one that had been produced from grapes that had been subjected to stress induced by low temperatures). Late-harvested grapes are exposed to very low temperatures by leaving them on the vines until very late in the year. This causes physiological changes within the plant and the production of substances that are believed to enhance the flavour of the wine. When plants become stressed or infected, concentrations of SA within them become increased and wine produced from plants infected with the fungus *Botrytis* might be expected to contain higher concentrations of SA than wines produced from uninfected plants. Two wines produced from grapes of plants infected in this way

Table XIV - The content of salicylic acid (SA) in soups

Manufacturer and type of soup	Organic or conventional ingredients	SA ng/g	Manufacturer and type of soup	Organic or conventional ingredients	SA ng/g	Manufacturer and type of soup	Organic or conventional ingredients	SA ng/g
1. **Suma** Carrot and Coriander	Organic	138	19. **Home Made** Lentil	Conventional	36	37. **Asda** Country Vegetable	Conventional	85
2. **Heinz** Mediterranean Tomato	Conventional	248	20. **Campbells** Tomato (batch 2)	Conventional	22	38. **Marks and Spencer** Carrot and Coriander (batch 2)	Conventional	20
3. **Grannys** Lentil and Bacon	Conventional	238	21. **Heinz** Lentil	Conventional	73	39. **Heinz** Vegetable	Conventional	29
4. **Go Organic** Tomato and Basil	Organic	8	22. **Heinz** Cream of Tomato	Conventional	9	40. **Campbells** Tomato and Red Pepper	Conventional	22
5. **Marks and Spencer** Mediterranean Tomato	Conventional	19	23. **Organic Soups** Cream of Tomato	Organic	22	41. **Heinz** Carrot and Coriander	Conventional	16
6. **Simply** Organic Mediterranean Tomato	Organic	54	24. **Asda** Smartprice Tomato	Conventional	5	42. **Heinz** Lentil	Conventional	158
7. **Baxters** Carrot and Coriander (batch 1)	Conventional	0	25. **Campbells** Mediterranean Tomato	Conventional	8	43. **Campbells** Tomato (batch 2)	Conventional	35
8. **Co-op** Tomato and Lentil	Conventional	23	26. **Heinz** Oxtail	Conventional	115	44. **Homemade** Chicken	Conventional	17
9. **Marks and Spencer** Carrot and Coriander (batch 1)	Conventional	21	27. **Baxters** Carrot and Parsnip with Nutmeg	Organic	120	A. **Heinz** Big Soup Chicken and Vegetable	Conventional	159
10. **Simply** Carrot and Coriander	Organic	1040	28. **Baxters** Tomato and Vegetable (batch 1)	Organic	134	B. **Safeway** Chunky Soup Vegetable	Conventional	64
11. **Campbells** Carrot and Coriander	Conventional	34	29. **Asda** Smartprice Cream of Chicken	Conventional	63	C. **Heinz** Thick Scotch Broth	Conventional	75
12. **Suma** Spicy Lentil	Organic	301	30. **Asda** Smartprice Vegetable	Conventional	60	D. **Safeway** Scotch Broth	Conventional	32
13. **St. Michaels** Tomato and Basil	Conventional	13	31. **Organic Soups** Lentil	Organic	117	45. **Baxters** Carrot and Coriander (batch 2)	Conventional	0
14. **Tesco** Red Pepper and Tomato	Conventional	8	32. **Organic Soups** Vegetable	Organic	63	46. **Tesco** Tomato (batch 2)	Organic	256
15. **Baxters** Traditional Tomato (batch 1)	Conventional	0	33. **Heinz** Mulligatawny Beef Curry	Conventional	>2000	47. **Baxters** Tomato and Vegetable (batch 2)	Organic	192
16. **Tesco** Tomato (batch 1)	Organic	234	34. **Heinz** Vegetable	Conventional	32	48. **Baxters** Traditional Tomato (batch 2)	Conventional	0
17. **Home Made** Scotch Broth	Conventional	6	35. **Campbells** Asparagus	Conventional	16	49. **Baxters** Mediterranean Tomato	Conventional	0
18. **Heinz** Cream of Chicken	Conventional	151	36. **Home Made** Broth	Conventional	160			

were examined. The concentrations of SA in this selection of wines are recorded in Table XV. The amount of SA in late-harvested wine (5.8 μg/100ml) and in wines that had been produced from the grapes of plants infected with *Botrytis* (4.7 μg/100ml and 6.4 μg/100ml) were almost two times higher than those present in all of the other wines. The median concentration in red wine produced from organically-grown grapes (1.9 μg/100ml) appears to be greater than the median concentration in red wine produced from conventionally-grown grapes (0.96 μg/100ml). However, a wide range of amounts of SA were present in the relatively few wines that were examined.

3.5.3. The content of SA in spices.

High concentrations of SA were found in sera from people living in a rural area of India who had been eating foodstuffs which were likely to have contained appreciable amounts of herbs and spices (see Table XVI). Although the content of SA in herbs and spices is controversial [96], some investigators have reported that they contain considerable amounts of the phenolic acid [75]. Therefore, a selection of spices frequently used in Indian cookery were examined for their content of salicylates. Table XVI shows the content of salicylates and SA found in these spices and some of the contents reported by earlier investigators.

Large amounts of salicylates were present in red chilli powder, cumin, paprika and turmeric. These four spices are widely used in substantial amounts in Indian cookery.

Table XV - The content of salicylic acid in various wines

	Concentration (μmol/l)	Concentration (μg/100ml)
White – conventional (produced from a variety of grapes from various countries)	0.17 0.48 0.08	1.2 3.3 0.6
White – organic (produced from a variety of grapes from various countries)	0.23 0.09 0.26	1.6 0.6 1.8
Red – conventional (produced from a variety of grapes from various countries)	0.14 0.06 0.41 0.37 0.10	1.0 0.4 2.9 2.6 0.7
Red – organic (produced from a variety of grapes from various countries)	0.27 0.22 0.31	1.9 1.5 2.1
White - late harvested	0.84	5.8
White - infected with *Botrytis*	0.67 0.97	4.7 6.4

The content of salicylic acid was determined according to the method of Blacklock et al [88].

Table XVI - The content of "total" salicylates (TS) and salicylic acid (SA) in various spices (mg/100g)

Spice	Source 1		Source 2		Reported previously A	Reported previously B		Reported previously C	Reported previously D
	TS	SA	TS	SA	TS	TS	SA	TS	TS
Asafoetida	3.8	0.5							
Cardamom (black)	27.0	<0.1							
Cardamom (green)	13.2	<0.1							
Chilli powder (red)	146.6	3.0							
Cinnamon	64.2	4.7	12.0	2.9	15.20	1.0		12.20	2.4
Cloves	2.5	0.4			5.74	2.0		Trace	
Coriander	2.7	0.8			0.20	0.1	Trace		
Cumin	1629.4	1474.7	980.0	744.9	45.00				
Fennel	2.0	<0.1			0.80			0.048	
Fenugreek	0.1	<0.1			12.20			0.298	
Garlic	5.6	<0.1			0.10			0.305	
Ginger	3.5	0.1			4.50				
Mustard	26.2	5.2			26.00	0.4	0.2		Trace
Paprika	104.3	3.0	104.7	10.8	203.00	0.7			0.3
Pepper seeds (black)	9.0	7.3			6.20	0.3			0.3
Tamarind	9.6	1.1							
Turmeric	350.5	23.3	288.9	15.3	76.40	1.7			

Source 1 : retailer supplying the Indian/Pakistani community; asafoetida, fennel and tamarind were imported from India.
Source 2 : "health food" shop
Previous authors : A Swain et al [75] B Herrmann [96]
C Variyar and Bandyopadhyay [77] D Venema et al [76]

The contents determined in this work were very much higher than those reported by Venema et al [76], Herrmann [96] and Variyar and Bandyopadhyay [77]. However, several of the values found were close to those reported by Swain et al [75].

3.5.4. The content of SA in cooked food

High concentrations of SA were found in sera from people living in a rural area of India who had eaten food containing appreciable amounts of herbs and spices. Chill powder, cumin, turmeric and paprika, which are used in appreciable amounts in Indian cookery, contained substantial amounts of salicylates (see Table XVI). In order to determine whether or not cooked food prepared by using such ingredients retained significant amounts of salicylates after the cooking process, the content of salicylates in four cooked vegetable dishes were determined (see Table XVII). Salicylates were present in appreciable amounts in all four dishes and the Vindaloo vegetable dish contained an amount of salicylates equivalent to that which is present in a low-dose aspirin tablet.

3.5.5 The bioavailablity of salicylates in cooked food

Having established that cooked foods of the type consumed by native Indians contained appreciable amounts of salicylates (see Table XVII) it was necessary to see if they were bioavailable.

A male volunteer, who had fasted for 10h, consumed a serving (545.3g) of vegetable vindaloo that contained 94.03 mg of total salicylates. Samples of blood

Table XVII - The content of salicylates and salicylic acid (SA) in cooked vegetable dishes

	Content of SA (mg/100g)	Content of salicylates (mg/100g)	Content of salicylates (mg/portion)
Vegetable curry [1] (Safeway)	0.18	5.6	12.0
Bengali vegetable curry [2] (Safeway)	0.28	6.2	18.1
Madras vegetable [3] (Jewel in the Crown Restaurant)	1.29	3.0	15.1
Vindaloo vegetable [4] (Jewel in the Crown Restaurant)	2.23	14.4	79.3

Ingredients

1 Onion, tomato, courgette, potato, carrot, beans, peas, cauliflower, spices, coriander, maize starch, garlic puree, tomato puree, ginger puree, onion seeds
33% vegetables (by wt).

2 Onion, tomato, courgette, potato, carrot, green beans, peas, aubergine, vegetable oil, cauliflower, spices, coriander, maize starch, garlic puree, tomato puree, ginger, peppers, cashew nut paste, spices, salt, sugar, mustard seeds, chilli, cumin seeds, onion seeds. 80% vegetables (by wt).

3 Traditional recipe

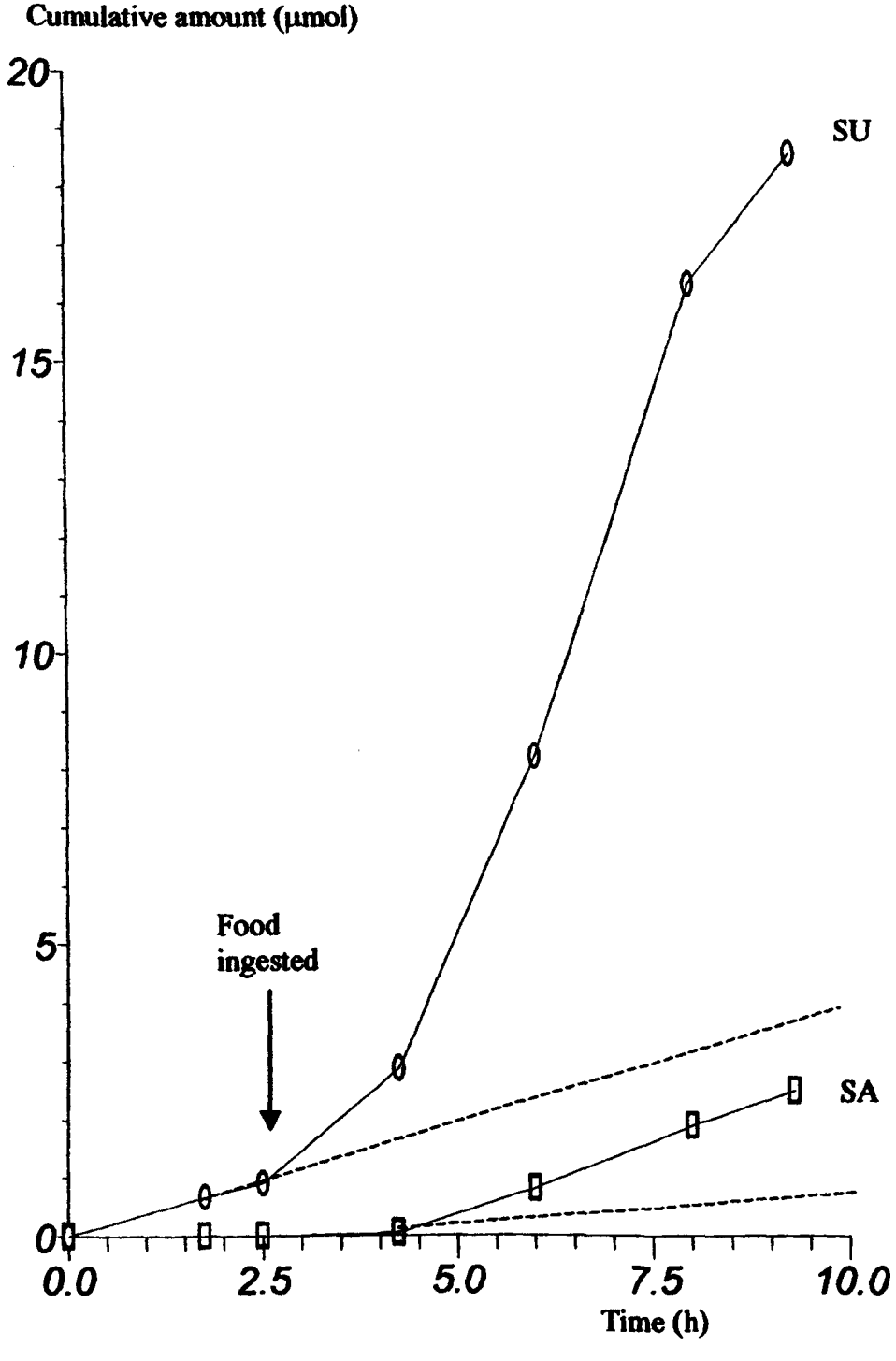
4 Traditional recipe

were withdrawn at approximately one hourly intervals throughout the timecourse of the investigation and the urine excreted was collected at regular intervals. The concentrations of SA in serum, and the concentrations of SA, SU and creatinine in the urine were determined (the SU to creatinine ratio being used to take account of variation in the subject's state of hydration during the course of the experiment). Figure 19 shows the cumulative amounts of SA and SU excreted in urine. The apparently linear portions of these plots observed prior to the ingestion of food indicates that there is a steady-state rate of excretion of SA and SU which is maintained by a process(es) other than the intake of salicylates from foodstuffs. Between 1 and 2 hours after taking the food containing salicylates, the concentration of SA in serum increased and about 4 hours after taking the food the ratio of SU to creatinine in urine had more than doubled (see Fig. 20). After the maximum concentration of SA in serum was attained, the subsequent decrease in the concentration of SA in blood was accompanied by the appearance of increased amounts of SU and SA in urine showing that the salicylates absorbed from food had been conjugated with glycine and excreted as SU or were excreted as unchanged SA. When the total amount of SA and SU excreted was expressed as a mol fraction of the salicylates that were present in the food it was found that at least 3% of the salicylates in the cooked food were bioavailable (absorbed, metabolized and excreted).

3.5.6 The concentration of SA in the blood of non-human animals.

The concentrations of SA and SU found in the sera and urine of people with different diets (see Tables VII and VIII) show that there is a relationship in man

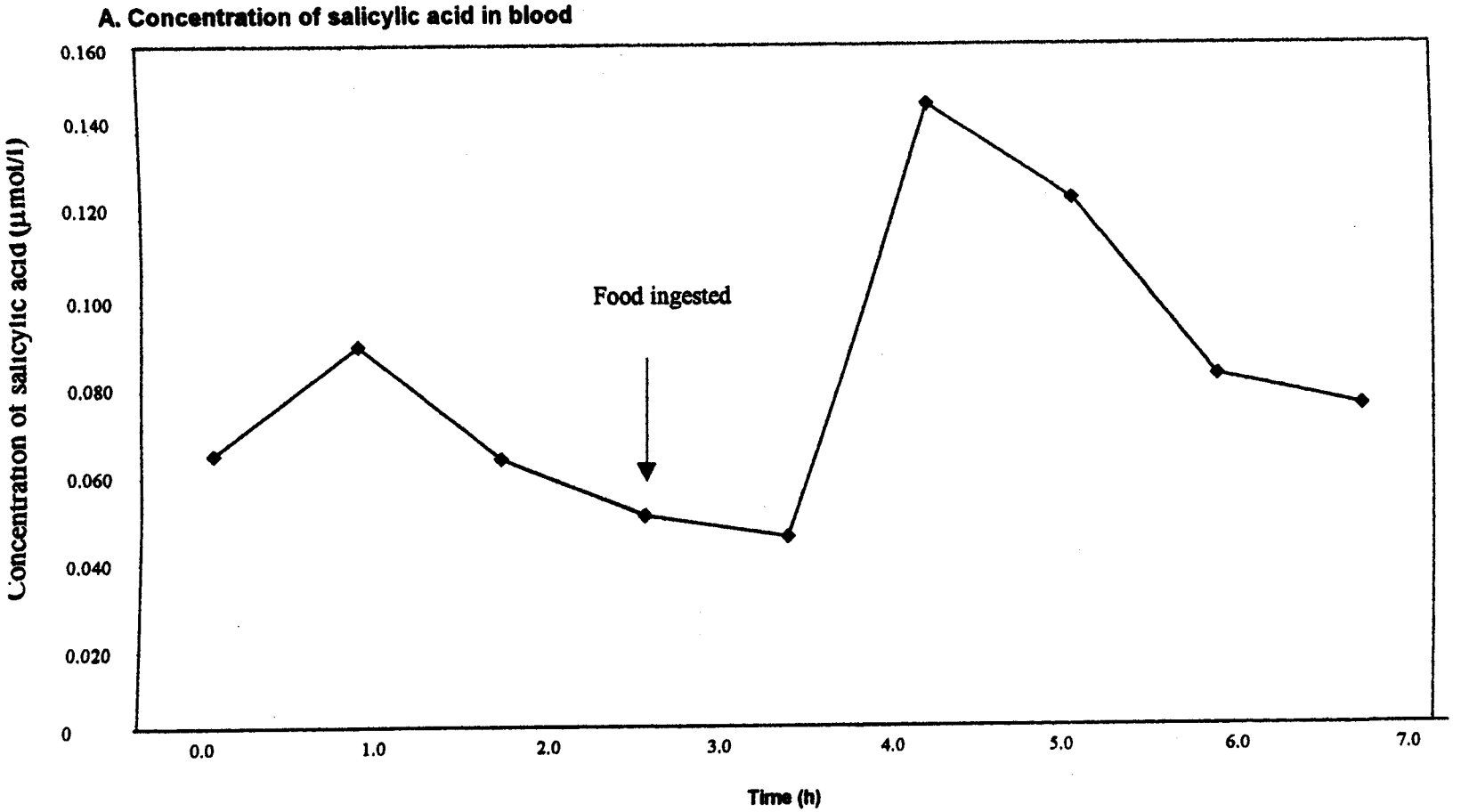
Figure 19. Bioavailability of salicylates in cooked food: Cumulative amounts of salicylic acid (SA) and salicyluric acid (SU) excreted in urine



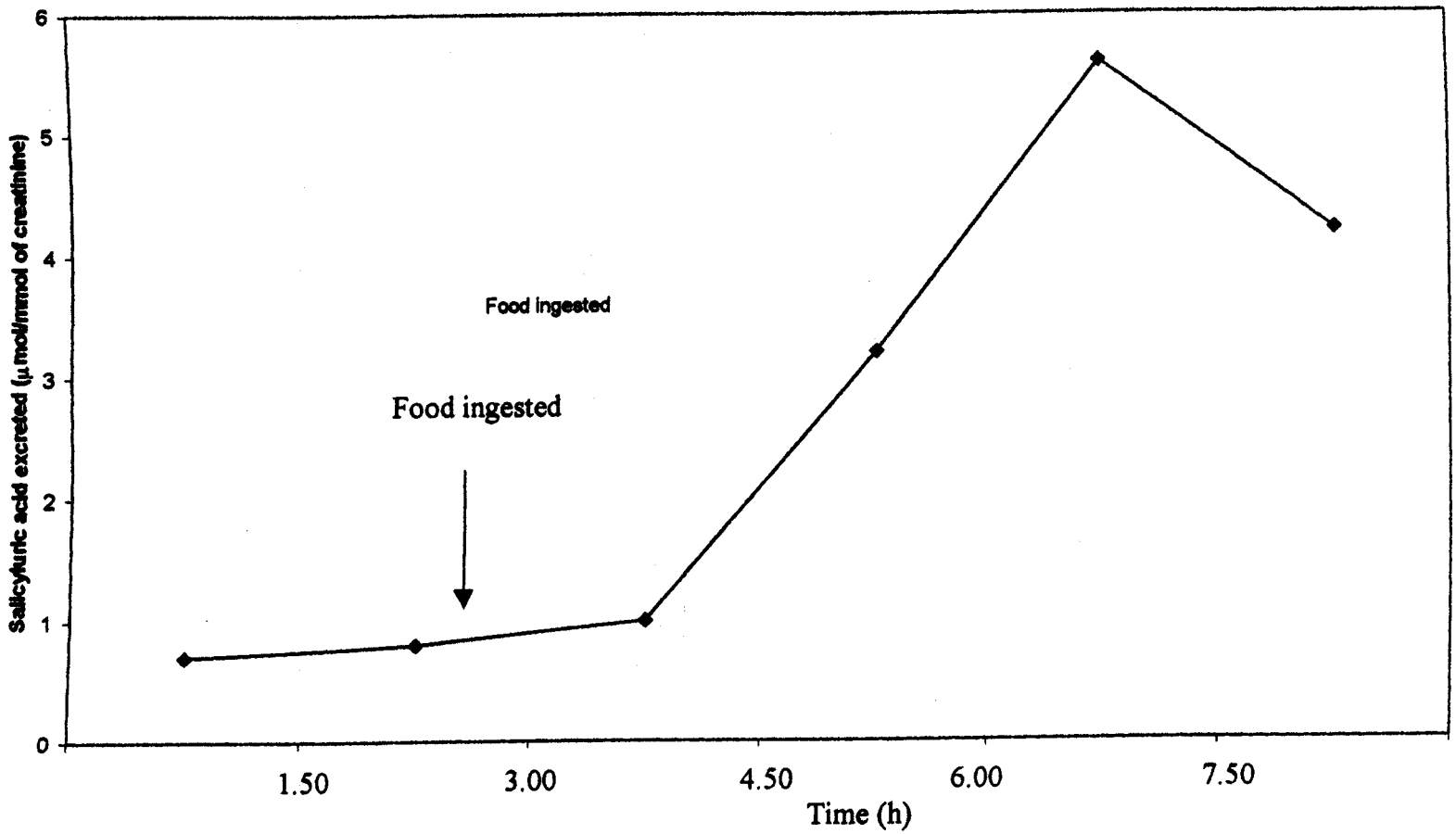
---------- The linear fits to the values observed prior to ingestion of food give the "intrinsic" rates of excretion.

Intrinsic rate of excretion of SA	= 2.0 µmol/24h (r^2 = 0.927)
Intrinsic rate of excretion of SU	= 8.9 µmol/24h (r^2 = 0.979)
Intrinsic rate of excretion of SA + SU	= 10.9 µmol/24h

Figure 20. The bioavailability of the salicylates in cooked food



B. Amount of salicyluric acid excreted (µmol/mmol creatinine)



between exposure to salicylates and diet. Sera and urine from vegetarians contained higher concentrations of salicylates than sera and urine from people with an unrestricted diet. Therefore, it might be illuminating to examine blood from a variety of animals so as to determine if SA is present and to see if there might be a relationship between the diet of the animal and the concentration of SA.

The concentration of SA in blood was determined by the method of Blacklock et al [88] and the values observed are recorded in Table XVIII. SA was present in the blood of all but two of these animals (crab and prawn); the highest concentration observed was in serum from a burrowing owl.

3.6. Other sources of salicylates

3.6.1 SA in the blood and SA and SU in the urine of a person consuming food devoid of SA

It has been suggested by several groups of workers that exposure to salicylates may not be due entirely to those in food [see e.g. 24,30]. Some of the SA found in the blood and urine of people who do not take salicylate drugs may be produced by the metabolic activity of gut bacteria, or by the transformation of precursors of SA present in the diet, or perhaps even by the action of endogenous enzyme systems. The steady excretion of SA and SU observed when dietary salicylates were excluded by fasting for 10h (see Fig. 19) supports this suggestion. To test these ideas a male volunteer who had not taken salicylate drugs for two weeks prior to the study was restricted to a diet of milk and water for a period of three days. Neither the milk nor the water contained SA. Under these conditions the

Table XVIII - The concentration of salicylic acid in the blood of a variety of animals

Animal	Phylogenetic class	Concentration of salicylic acid (μmol/l)	Animal	Phylogenetic class	Concentration of salicylic acid (μmol/l)
Burrowing owl	Aves	9.854	Giant anteater	Mammalia	0.293
Ne-ne	Aves	5.609	African lion	Mammalia	* 0.226
Indian rhinoceros	Mammalia	4.700	Cow	Mammalia	0.216
Pygmy hippopotamus	Mammalia	2.384	Gelada baboon	Mammalia	0.210
Agouti	Mammalia	2.116	Collared peccary	Reptilia	0.237
Asian elephant	Mammalia	1.635	Chinese alligator	Reptilia	0.156
Burmese python	Reptilia	1.362	Domestic cat	Mammalia	0.144
Rabbit	Mammalia	1.129	Pond heron	Aves	0.136
Piglet	Mammalia	1.010	Gorilla	Mammalia	0.125
Arabian oryx	Mammalia	0.777	Red faced spider monkey	Mammalia	0.080
Sheep	Mammalia	0.715	Mouse	Mammalia	0.078
Tiger	Mammalia	* 0.661	Rat	Mammalia	0.069
Brown trout	Pisces	0.538	** Domestic cat	Mammalia	0.058
Giraffe	Mammalia	0.507	Chimpanzee	Mammalia	0.033
Donkey	Mammalia	0.473	European shore crab	Crustacea	< 0.005
Sacred Ibis	Aves	0.353	Prawn	Crustacea	< 0.005
Goat	Mammalia	0.310			

* Mean concentration in the blood of 5 animals

** Domestic cat fed meat only.

Limit of detection in blood, 0.005μmol/l

person did not receive SA from food and almost all of the SA that was present in his blood at the start of the investigation should have been eliminated within the first day of consuming the restricted diet. (SA in blood has a half-life of 2 – 4h [97]).

Blood was withdrawn from the subject at 12 hourly intervals and its concentration of SA changed very little over the 72h of the experiment (see Fig. 21a). Urine from the experimental subject was collected over 6 consecutive 12h time periods. SA and SU were present in all 6 fractions of urine. A plot (see Fig. 21b) of the cumulative amount of SA and SU excreted was linear and indicated a rate of excretion of (SA + SU) of ~ 2.1 μmol/24h. These data suggest that another source(s) of SA or other process(es) were responsible for the steady rate of excretion of SA and SU by this individual throughout the timecourse of this experiment.

3.6.2. The contribution of potential precursors of SA to the SA in people who do not take salicylate drugs

Several compounds might be converted into SA and contribute to the concentrations of SA in the serum of the people who had not taken salicylate drugs. Benzoic acid, chorismic acid and *o*-coumaric acid are possible precursors of SA that are present in foodstuffs (see the Introduction). Compounds with the R_t of chorismic acid and *o*-coumaric acid were detected when extracts of serum were chromatographed according to the method of Blacklock et al [88].

As a means of tentatively identifying these compounds, the chromatographic

Figure 21 The effect of eliminating dietary salicylic acid on (a) the concentration of salicylic acid (SA) in blood and (b) the cumulative amount of salicylates (SA + SU) excreted

Figure 21a

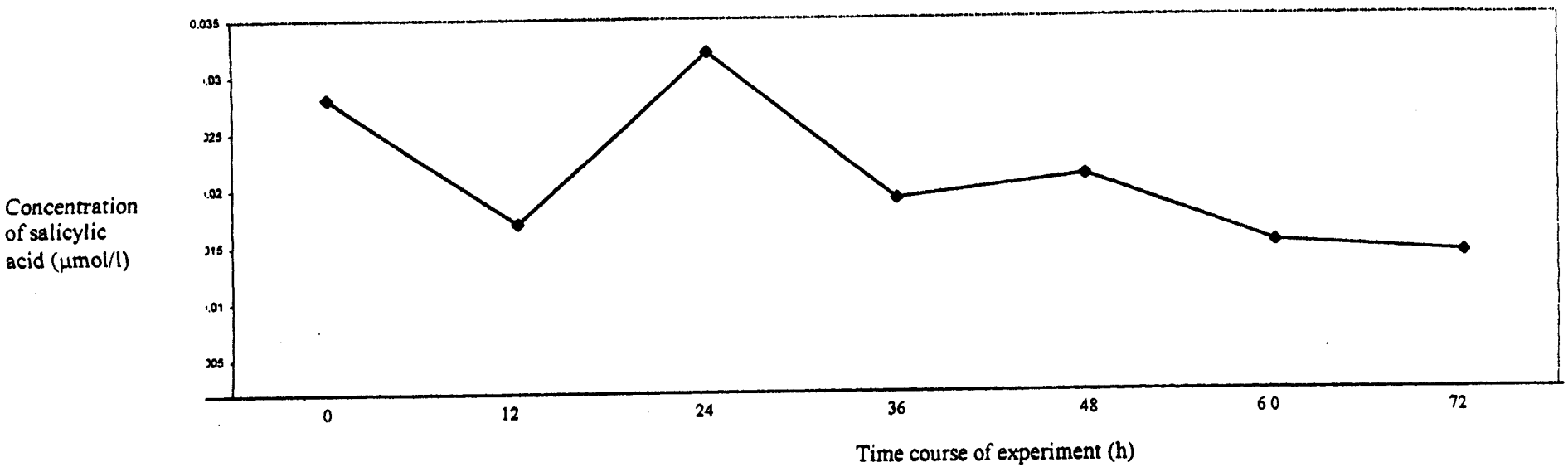
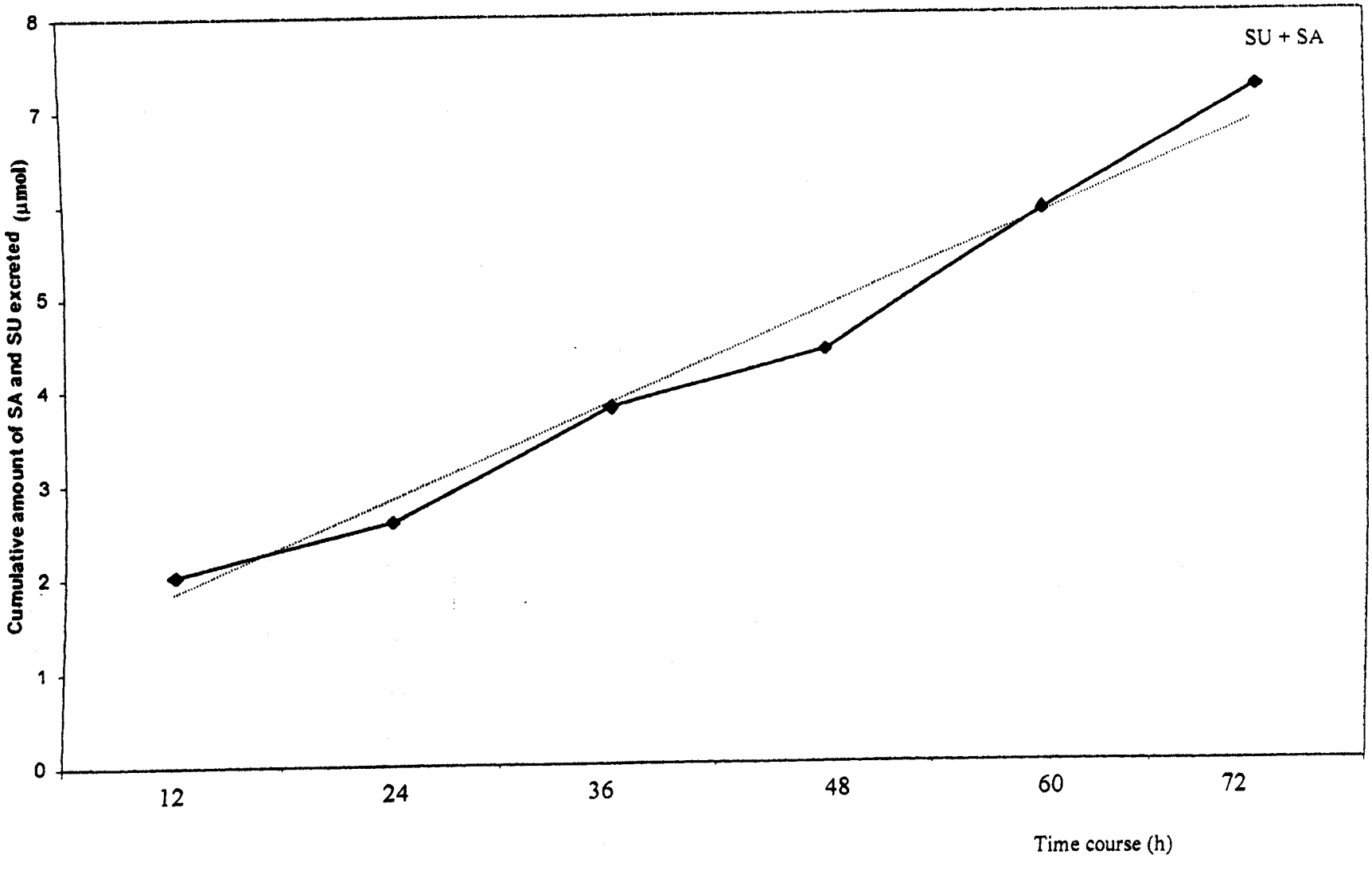


Figure 21b



--------- Best linear fit assuming equal variance of the individual values on the ordinate axis.
Intrinsic rate of excretion of (SA + SU) = 2.1 µmol/24h (r^2 = 0.96)

conditions were varied. An isocratic procedure employing only mobile phase A (50% citrate buffer: 50% methanol v/v) was tried, as was the method of Blacklock et al [88] but with the rate of elution reduced to 0.3 ml/min. When these chromatographic conditions were used a compound extracted from acidified serum with ethyl acetate was eluted at the R_t of chorismic acid. Under isocratic conditions, a compound extracted from serum was eluted at the R_t of *o*-coumaric acid. However, when the rate of elution was decreased during Blacklock's scheme of stepwise elution none of the compounds eluted had the R_t of *o*-coumaric acid (see Table XIX).

A potential precursor of SA that is present in foods from plants and that also is added to preserve many foodstuffs is benzoic acid; it may become hydroxylated *in vivo* to generate SA. To see if benzoic acid might be converted into SA in man, sodium benzoate was administered orally to two male volunteers who had not taken salicylate drugs for 2 weeks prior to the investigation and who had consumed food from the same batches at the same time and in the same quantities for four days (see section 2.2.3a). On the third and fourth days one volunteer took 2.0g of sodium benzoate and the other took 1.0g. Samples of blood were withdrawn on each of the four days of the investigation and also on the morning of the fifth day. Urine was collected during the 4 consecutive 24h time periods of the study.

The concentration of SA in the blood of each of the subjects varied very little throughout the time course of the experiment (data not shown). However, the

Table XIX - The retention times, under various conditions of elution, of chorismic acid, *o*-coumaric acid and compounds extracted from serum

Compound	Retention time (min)		
	Conditions of elution described by Blacklock et al [88]	Isocratic elution	Conditions of Blacklock et al [88] but with a reduced rate of elution
Chorismic acid	16.00	4.52	36.42
Compound extracted from serum	15.98	4.49	36.36
o-coumaric acid	46.55	35.23	53.21
Compound extracted from serum	46.50	35.17	No compound eluted close to R_t = 53.21

See the text.

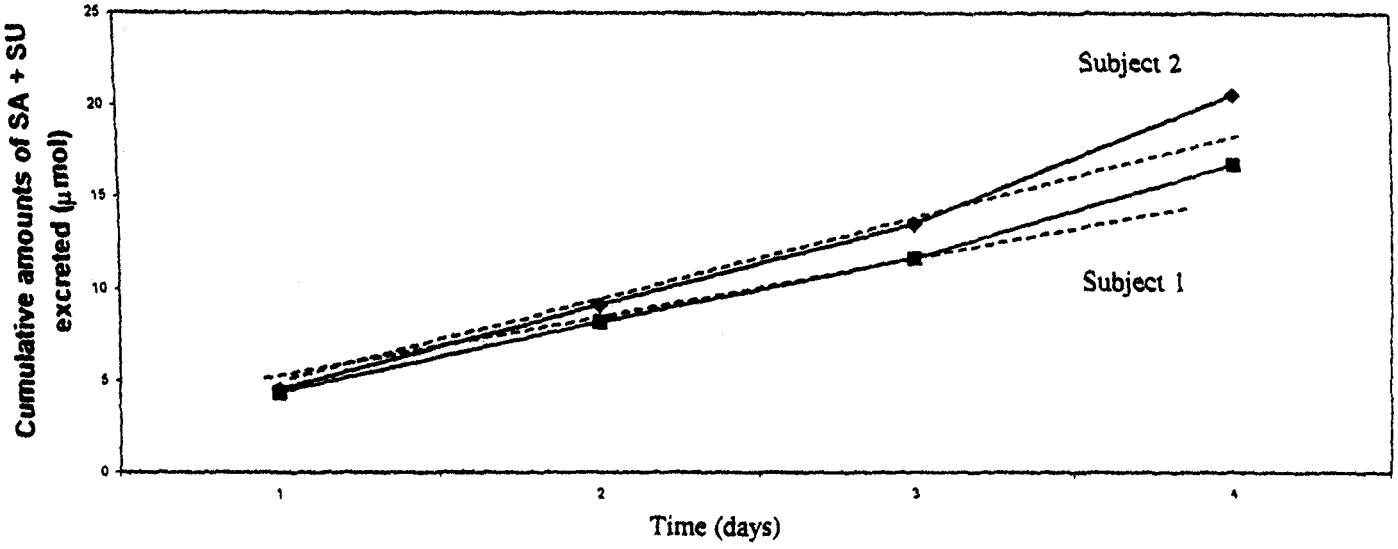
cumulative amounts of SA and SU excreted in urine by both subjects increased linearly showing rates of excretion prior to the administration of benzoate of 3.47 and 4.38 μmol/24h. However, on day 4, i.e. on the day after they had taken sodium benzoate for the first time (see Fig. 22) these rates of excretion were increased to 5.0 and.7.02 μmol/24h respectively. This increase was significant ($p < 0.001$, Mann-Whitney U-test) for only one of the subjects (subject 2, who had been given the higher dose of sodium benzoate). These findings indicate that benzoic acid present in food might be transformed into SA in man.

3.6.3. Gut bacteria and their contribution to the SA in the serum of animals who had not received salicylate drugs.

Certain enteric bacteria synthesize and dissimilate SA, and they might have generated some of the SA in individuals who had not taken salicylate drugs.

The administration of antibiotics to animals alters the number and composition of intestinal microflora [98]. When animals are treated with antibiotics for more than four or five days their intestinal microflora are altered in composition and reduced in number. These animals have been described as "germ-free". Animals that have been surgically removed from the womb and then reared under sterile conditions and fed sterile food so as to prevent the development of intestinal microflora have also been described as "germ-free". Investigations using blood from these two types of "germ free" animals and blood from ordinary animals of the same strain (with an established gut microflora) were made to see if there was any difference in the concentrations of SA present in serum.

Figure 22. The influence of oral administration of sodium benzoate on the amounts of salicylic acid (SA) and salicyluric acid (SU) excreted



---------- Best linear fit to the values observed prior to administration of benzoate assuming equal variance of the individual values on the ordinate axis.

Subject 1, rate of excretion of (SA + SU) = 3.47 μmol/24h (r^2 = 0.987)
Subject 2, rate of excretion of (SA + SU) = 4.38 μmol/24h (r^2 = 0.996)

In the first part of the investigation, to determine the possible contribution of gut microflora to the concentration of SA in serum, the concentration of SA was determined in the combined sera of six "germ-free" mice that had been treated with antibiotics and in the combined sera from six mice of the same strain that had not. The mice that had been treated with antibiotics had a slightly higher concentration of SA in serum (0.309 μmol/l) than the animals that had not (0.268 μmol/l).

In the second part of the investigation, the concentration of SA was determined in blood collected from two groups of rats. One group (n = 8) had been delivered by Caesarian section, fed sterilized food and reared in a sterile environment. The other group (n = 8) contained animals of the same strain, that had been born naturally, fed sterilized food and reared under standard laboratory conditions. Their food did not contain SA. The concentration of SA was approximately 2.5 times greater in the combined sera of the "germ-free" animals (0.166 μmol/l) than in that of the animals of the control group (0.069 μmol/l).

From the results of both of these experiments it can be seen that the concentrations of SA in serum were lower in animals with an established gut microflora. These findings suggest that the metabolic activities of intestinal microorganisms might not be involved in the formation of the SA that occurs in the blood of these animals.

4.0 DISCUSSION

SA, its phenyl glucoside and its carboxyl esters are constituents of many plants [9]. Therefore, it is reasonable to expect that salicylates are present in food prepared from plant materials. An oxidisable compound with the R_t of SA was observed when extracts of soup, wine, spices and cooked food were chromatographed using the method of Blacklock et al [88], which had been developed to separate SA and its hydroxylated metabolites (see e.g. Fig. 18). Alteration of these chromatographic conditions resulted in alterations of the R_t of the substance extracted from wine and soup that were very similar to those they produced in the R_t of SA (see Fig. 13).

When urine from 27 healthy volunteers, who had not taken salicylate drugs, was acidified, extracted with ethyl acetate and the compounds removed were separated using HPLC, it was noted that two of the oxidisable compounds detected had R_t that were very similar to those of SA and SU (see Fig.7). In addition, when the chromatographic conditions were varied, the R_t of the compounds suspected to be SA and SU were altered in a very similar way to the alterations observed in the R_t of the authentic compounds (see Fig.8).

However, urine and foodstuffs contain many hydrophobic compounds that partition into ethyl acetate from acidified aqueous phases (see Results 3.1.1) and some of them might have chromatographic characteristics that are similar to those of the salicylates under investigation. Moreover, variation of chromatographic characteristics produced by adjustments to the conditions of elution does not

provide conclusive proof of the identities of the two compounds from urine and the compound extracted from foodstuffs.

Salicylate hydroxylase has restricted substrate specificity for *o*-hydroxybenzoic acids and it catalyses the oxidative decarboxylation of SA most rapidly [91]. Treatment of urine with salicylate hydroxylase caused the compounds suspected of being SA and SU to be transformed (see Fig. 9). Thus, evidence from enzymic and chromatographic studies suggested strongly that SA and SU were present in the urine of people who had not taken salicylate drugs. When an extract of soup was treated with salicylate hydroxylase the unknown compound was transformed (see Fig.13). This circumstantial evidence suggested strongly that SA also may be a constituent of certain foods.

Examination by GCMS showed that the gas chromatographic R_t of two of the unknown compounds extracted from urine (after esterification) were very similar to the gas chromatographic R_ts of methyl salicylate, SA treated with acetyl chloride in methanol, and SU treated with acetyl chloride in methanol (see Results 3.4.1, Fig.10 and Table IV). The mass spectra of these esterified compounds were compared with those of compounds held in the Pfleger, Maurer and Weber library (stored in the computer attached to the mass spectrometer) [92]. A programme within the computer's software allows comparison of the spectra of unknown compounds with those held in the library, and, if and when a match is found, the identity of the unknown substance is printed along with an estimate of the probability of the identification being correct. This process showed that the esters of the compounds extracted from urine were most likely to be methyl salicylate

and methyl salicylurate with probabilities of 81% and 80% respectively. The probabilities recorded for methyl salicylate, for SA treated with acetyl chloride in methanol and for SU treated with acetyl chloride in methanol were 96%, 94% and 92% respectively.

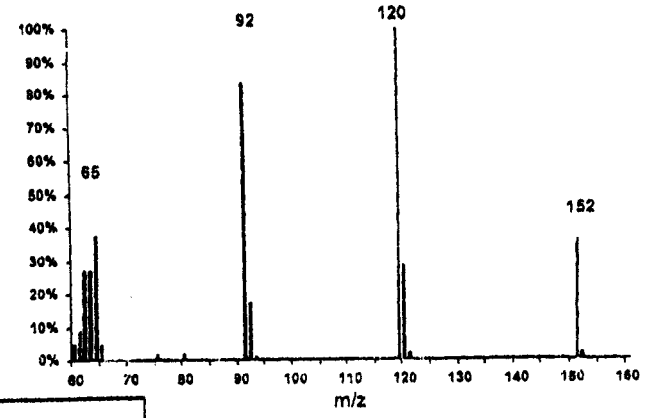
Extracts of a soup (Co-op Tomato and Lentil) and of three spices (cumin, turmeric and paprika) also were treated with acetyl chloride in methanol and examined by using GCMS. One of the compounds separated from these treated extracts had a gas chromatographic R_t and a mass spectrum very similar to those of methyl salicylate (see Fig. 14 and Table IX). When these were compared with spectra of known compounds it was found that the spectra of the unknown compound in soup, turmeric, cumin and paprika after esterification matched that of methyl salicylate with probabilities of 95%, 94%, 78%, and 85% respectively.

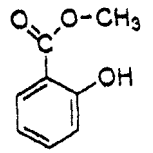
Figure 23 shows the mass spectrometric fragmentation patterns of methyl salicylate and methyl salicylurate reported by Pfleger, Mauer and Weber [92]. These match very closely the fragmentations observed for the esters produced from the compounds extracted from soup, urine and spices (see Tables IV and IX - XII and Figs 10 and 14 - 17).

From all of these results it was concluded that SA and SU are components of the urine of people who had not taken salicylate drugs, and that SA was a constituent of soup, wine and spices.

Figure 23 Mass spectrometric fragmentation pattern of (A) methyl salicylate, (B) methyl salicylurate (Pfleger et al [92])

A





	Mass
Principal ion	152
Base peak	120 (most abundant)
Fragment	92
Fragment	65

Fragmentation of molecular ion radical → Cation + radical

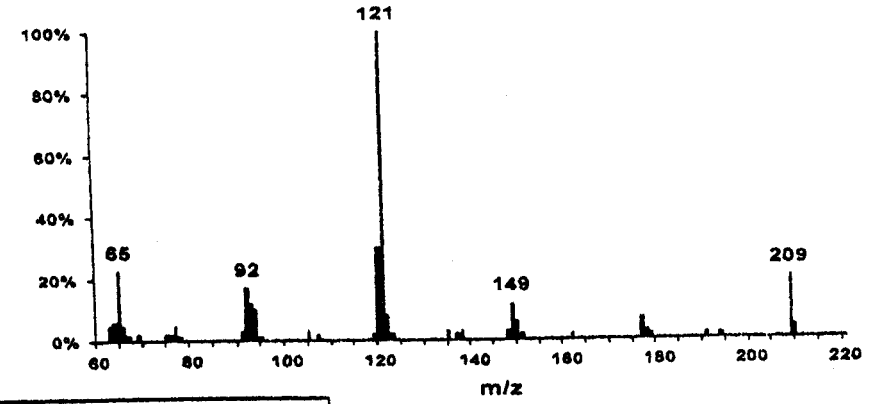
Fragmentation of molecular ion radical → Radical cation + neutral fragment → Cation + radical

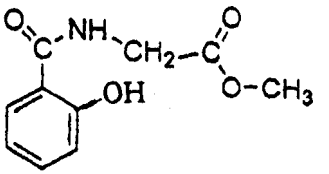
152 – 32 = 120
A loss of 32 represents the removal of
OH^- group (17)
CH_3 group (15)

152 – 60 = 92
A loss of 60 represents the removal of
$COOCH_3$ group (59)
H^+ (1)

A loss of 65 represents the fragment
C_5H_5 (65)

B.





	Mass
Principal ion	209
Base peak	121 (most abundant)
Fragment	149
Fragment	92
Fragment	65

Fragmentation of molecular ion radical → Cation + radical

Fragmentation of molecular ion radical → Radical cation + neutral fragment → Cation + radical

209 – 88 = <u>121</u>

A loss of 88 represents the removal of the

$COOCH_3$	group	(59)
CH_2	group	(14)
NH	group	(15)

209 – 117 = <u>92</u>

A loss of 117 represents the removal of the

$COOCH_3$	group	(59)
CH_2	group	(14)
NH	group	(15)
CO	group	(28)
H^+		(1)

209 – <u>60</u> = 149

A loss of 60 represents the removal of the

$COOCH_3$	group	(59)
H^+		(1)

A loss of 65 represents the fragment C_5H_5 (65)

Once reliable quantitative methods for determining SA in foods and for SA and SU in urine were available, it might be possible to measure the extent of exposure to salicylates and to see if there might be a correlation between this and the incidence of certain diseases. A search of the literature was made to discover if any previous investigators had developed a suitable method for the determination of SA and SU in urine from people who had not taken salicylate drugs so that it might be used to investigate the exposure of various groups of people to salicylates. This revealed that most of the published methods had been devised to measure these two compounds in the urine of people and animals that had been given salicylate drugs [101]. Many of the methods were used in the study of the pharmacokinetics of aspirin [102, 103]. Other methods, for example the colorimetric method of Trinder (see Weiner [104]) and the polarized fluorescence immunoassay produced commercially by Abbott Laboratories (see Cullen [105]), had been developed to determine the concentrations of salicylates in serum of people after they had taken massive (possibly toxic) doses of aspirin.

Several of these studies [101 – 103, 106, 107] involved the determination of the concentrations of three of the metabolites of aspirin, namely SA, 2,3-DHBA, and 2,5-DHBA in plasma obtained from people who had received aspirin at doses between 100mg and 1200 mg per day. In a similar study McCabe et al [108] used a chromatographic method with isocratic elution to separate SA, 2,3-DHBA and 2,5-DHBA extracted from tissues of two groups of rats. The compounds were determined electrochemically and the concentrations of the hydroxylated products (2,3-DHBA and 2,5-DHBA) were used to assess the extent of production of the

hydroxyl radical (OH˙). Some of the rats had been given SA (100 mg/kg) prior to examination of their tissues, and, under these conditions, McCabe et al observed concentrations of SA in liver, kidney, and brain in the range 0.03 – 0.3 μmol/g. However, they were unable to detect SA in any of these tissues from animals that had not been given SA. In a similar study, Sloot et al [109] reported that SA was present in the striatum and cerebro-spinal fluid of rats that had received a higher dose of SA (300mg/kg). Sloot et al could not detect SA in tissues from animals that had not been given SA prior to investigation. Moreover, they did not establish conclusively that the compound detected in tissues from animals that had been given SA was indeed SA; they observed that the compound's chromatographic R_t was similar to that of SA.

Blacklock et al [88] developed a sensitive and reliable method, employing HPLC with electrochemical quantification, to determine the concentrations of SA and its hydroxylated derivatives in serum from people who had taken aspirin (75 mg per day) for a considerable period of time. Interestingly, Paterson et al [30], by applying this method, also observed small amounts of compounds with R_t similar to those of SA, 2,3- and 2,5-DHBA in serum from people who had not taken aspirin and from the results of chromatographic and electrochemical tests, suggested that these compounds were SA, 2,3-DHBA and 2,5-DHBA. However, their attempt to confirm the identity of the substance in serum thought to be SA using the derivatising agent N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and GCMS was not entirely conclusive. MSTFA decomposed under the conditions of derivatisation and produced a variety of siloxanes that could not be

separated completely from the TMS_2 derivative of SA. The results of the present work using a simple esterifying agent (acetyl chloride in methanol) are much more convincing (see Fig.10 and Table IV).

The quantitative method of Blacklock et al [88] also was tried to see if it was suitable for the determination of SA and SU in urine and for SA in food. Blood and urine contain different mixtures of compounds that partition into ethyl acetate from acidic aqueous phases. Numerous compounds extracted from urine with ethyl acetate had chromatographic characteristics that were very similar to those of the determinands and they were incompletely resolved from SA, SU and the internal standard (4-methylsalicylic acid) [see Figs.6 and 7]. However, the method was suitable for the determination of SA in foodstuffs (see Fig. 18). After the accuracy and precision of this method were examined (see Table XIII and section 3.4.2a) it was obvious that the method was more accurate and reliable for the measurement of the small amounts of SA in foodstuffs than those previously reported. Many investigators of the content of salicylates in foodstuffs did not examine thoroughly the precision and accuracy of their methods. For example, Venema et al [76] reported that some of their duplicate values were in poor agreement and they quoted an inter-assay coefficient of variation of 8%.

Studies of the concentrations of the salicylates present in urine from people who had not taken salicylate drugs frequently lack data concerning reproducibility, repeatability and/or identification of the substances being measured [24,26 –27, 80, 110]. In 1956 Armstrong et al [24] published results that indicated that SA and SU might have been present in urine from people who had not taken salicylate

drugs. They used 2-dimensional paper chromatography and the presence of small amounts of a compound that was believed to be SU was recorded. They suggested that salicylates or a precursor(s) of SA might have been present in food and that the actions of gut bacteria might have produced SA from precursors.

Although these workers [24] suggested that foodstuffs were a possible source of salicylates no further investigations of salicylates in people who had not taken salicylate drugs was made until 1964 when von Studnitz [26] investigated the effect of a chemically-defined diet on the urinary excretion of minerals and aromatic compounds by people ingesting only glucose and water. These workers [26] employed 2-dimensional paper chromatography also and, although they did not identify or quantify all of the compounds in urine, they suggested that SU might be a constituent of the urine of people with this restricted diet.

Chen et al [111] suggested that SA and SU were present in the urine of ruminants. Their method involved separation of the compounds in urine by using HPLC with a programme of gradient elution. Ultraviolet absorption was used to measure the concentrations of both SA and "total" salicylates (SA plus conjugated forms) and they recorded concentrations of about 0.1mmol/l of SA and SU. However, SA had not been separated completely from phenol and they were unable to obtain reliable values for the content of SA and SU.

Although several groups of investigators, including Armstrong et al [24] and Young [27], had speculated that salicylates might be present in urine from people who had not taken salicylate drugs, they did not quantify them. Other investigators who quantified these compounds in urine, did not establish their

identities [26, 80]. Therefore, if the exposure of people to salicylates were to be investigated, it would be necessary to develop an accurate, sensitive and reliable method to determine the concentrations of SA and SU in urine not only from people who did not take salicylate drugs but possibly also from people who had restricted "salicylate-free" diets. The method required for this study would have to be sensitive, accurate and reliable if the very low concentrations of SA in biological fluids and foodstuffs were to be measured with confidence. Blacklock et al [88] found different ranges of concentrations of SA in serum from people who had not taken salicylate drugs and had an unrestricted diet (0.020 – 0.20 μmol/l) and in serum from a group of strict vegetarians (0.040 – 2.47 μmol/). The range recorded for the group of vegetarians was overlapped with the range of concentrations found in the serum of people who had taken a low dose of aspirin (75mg/day) regularly. Therefore, it is entirely likely that a wide range of concentrations of SU and SA would be present in the urine of these groups of people, and it is likely that the concentration of SU would be much higher than that of SA; SU is the main urinary metabolite of SA [99]. Therefore, a trustworthy method to determine these substances would need to be accurate throughout considerable ranges of concentrations of SA and SU.

Since a suitable method for the determination of SA and SU in urine was not available it was decided to investigate the quantitative method of Blacklock et al [88] to see if it might be adapted and applied to urine. Blacklock et al extracted acidified plasma with ethyl acetate and separated the solutes removed by using HPLC with stepwise gradient elution. However, their programme of elution did not achieve a clean separation of SA, SU and the internal standard from the other

compounds that were extracted (see Table I and Fig. 6) and it was necessary to alter the chromatographic conditions to see if complete resolution of the determinands could be achieved.

When isocratic conditions were employed using each of the mobile phases (mobile phase A contained 50% methanol in citrate buffer, pH 4.0; mobile phase B contained 5% methanol in citrate buffer, pH 3.8), many of the compounds (including SU) extracted from urine with ethyl acetate were not resolved. When mobile phase C (citrate buffer, pH 3.8) was substituted for mobile phase B in the programme of gradient elution employed by Blacklock et al [88] the resolution of SU was improved without having a detrimental effect on the resolution of either SA or 4-methylsalicylic acid (see Fig. 7). A much better separation of the determinands was achieved when the programme of gradient elution was modified. A series of changes to the proportions of mobile phase A and mobile phase C gave increasingly improved resolutions of the determinands (see Fig. 8). A decrease in the rate of elution prior to the elution of SU resulted in the satisfactory separation of SA and SU. However, this change to the rate of elution resulted also in a change of R_t of the internal standard so that its separation deteriorated. Therefore, it was necessary to employ an additional decrease in the rate of elution just prior to the R_t of the internal standard so as to restore its complete separation and to develop a scheme that allowed satisfactory resolution of SA, SU and 4-methylsalicylic acid from each other and from the other neutral and acidic hydrophobic compounds extracted from urine (see Fig. 8 and Table II).

Having established that there were good linear relationships between the ratio of the detector response due to the determinands to that due to the internal standard and the concentrations of these determinands (see Figs. 11,12) the reliability and accuracy of this method were examined. The limit of detection of the method (determined as described in the Materials and Methods, section 2.3.8.) was 0.005 μmol/l for both SA and SU in urine. During the course of this work the lowest concentrations of SA and SU reported in urine were 0.014 μmol/l and 0.011 μmol/l respectively. In four samples of urine, however, the concentrations of SA and SU were lower than 0.005 μmol/l and these were reported as less than the limit of detection (see Table VII).

Electrochemical oxidation was used in the quantification stage of the method developed. Several factors might influence the extent of oxidation of the determinands prior to the determination and these include exposure to air, increased temperatures and the promotion of oxidation caused by metal ions. A variety of precautions were taken to minimize the oxidation of SA and SU prior to quantification. Urine was stored in tightly sealed, well-filled containers and these were stored at – 20°C or –70°C prior to evaluation. During manipulation prior to chromatography the opportunity for oxidation was reduced by using screw-capped bottles and by the addition of a chelating agent (EDTA) to the extraction mixture and to the solvent used for reconstituting the dried extracts (see Materials and Methods).

To assess the stability of SA and SU in urine during collection and throughout a 24h storage period, the concentrations of these two compounds were determined

immediately after collection and after urine had been stored for 24h at room temperature, at 4°C, and at –20°C. The concentrations of SA and SU found were not statistically different; the variation noted was similar to the CV of the inter-assay precision (see Table VI). These results show that SA and SU in urine did not decompose significantly.

The method of Vree et al [112] for determining the concentrations of compounds in urine that they believed were SA and SU had limits of detection of 5.0 μmol/l for both SA and SU. For most previously published methods, however, the limit of detection was not recorded. The sensitivity of the method developed in this work (0.005 μmol/l for both SA and SU) shows that it is 1000 times more sensitive than that of Vree et al [112]. Several methods of extracting salicylates from urine have been utilised but few of them describe the efficiency of this process. Hutt et al [113] treated acidified urine with diethyl ether and they achieved a mean efficiency of extraction of salicylates of 68%. Janssen et al [80], who also used ether to extract the salicylates, reported that 80% of the salicylates in urine were extracted. In this work a more suitable and more polar solvent, ethyl acetate, was used and higher efficiencies of removal of SA (85%) and SU (87%) from urine were obtained.

Table XX shows the CVs for intra-assay and inter-assay precision of this method and previously published values. These data show that this method is more reliable and accurate than those of Vree et al [112] and Liu and Smith [99] (see Table XXI). It was concluded that the method was sufficiently sensitive, accurate and reliable for the determination of SA and SU in urine, and when used in

Table XX - Values of inter-assay and intra-assay precision of various methods for the determination of salicylic acid (SA) and salicyluric acid (SU) in urine

	Intra-assay (CV%)		Inter-assay (CV%)	
	SA	SU	SA	SU
This work[3]	0.03	0.07	2.48	2.43
Vree [1]	1.78	1.13	5.70	6.10
Liu [2]	2.76	3.30	8.00	4.13
	Total salicylates		Total salicylates	
Janssen [4]	2.0		9.0	

1 Reference [112]
2 Reference [99]
3 See the results in Table V
4 Reference [80]

Table XXI Other characteristics of the various methods for determining salicylic acid and its metabolites in urine

Method of	Chromatographic Elution	Method of Quantification	Determinand	Limit of detection /quantification* (μmol/l)
Liu and Smith (ref. 99)	isocratic	ultraviolet absorption	2,5-dihydroxybenzoic acid	3.2
			salicylic acid acyl glucuronide	0.6
			salicyluric acid	1.0
			salicylic acid	NR
Vree et al (ref.112)	linear gradient	ultraviolet absorption	salicyluric acid phenolic glucuronide	14*
			salicylic acid phenolic glucuronide	15*
			2,5-dihydroxybenzoic acid	32*
			salicylic acid acyl glucuronide	15*
			salicyluric acid	25*
			salicylic acid	36*
Janssen et al (ref. 80)	isocratic	fluorescence emission	salicylic acid	0.1
This work	stepwise gradient	electrochemical oxidation	salicyluric acid	0.005
			salicylic acid	

The asterisk indicates a value of limit of quantification. NR indicates that the value was not recorded.

conjunction with the method of Blacklock et al [88] for the determination of SA in blood and foodstuffs, these methods would enable the investigation of exposure to salicylates.

There is no doubt there is up to a 50% reduction in the incidence of colorectal cancer among people who take aspirin regularly [115,116] and that populations that have a diet that is rich in fruits, vegetables, herbs and spices, that contains appreciable amounts of SA, experience the lowest incidences of colorectal cancer in the world [58]. Perry et al [117] suggested that the reduction in the chance of people developing cardiovascular disease is related to their consumption of food that contains naturally-occurring salicylates. It has been suggested also that the negative correlation between the extent of consumption of red wine and the risk of experiencing a myocardial infarction is due to the content of salicylates in wine [118]. The World Health Organization recommends that people should eat at least "5 portions of fruit and vegetables per day", the recommendation being based on the large body of evidence showing that there is a negative correlation between the content of dietary fruit and vegetables and the incidence of colorectal cancers [58]. The results reported in this thesis suggest that SA is the chemoprotective substance. Several groups of workers [24, 80, 88] have proposed that salicylates in food might contribute to the amount of salicylate present in blood and urine from people who had not taken salicylate drugs. Blacklock et al [88] concluded that foodstuffs derived from plants are major sources of SA. Therefore, it is reasonable to suggest that people who consume foods that contain appreciable

amounts of salicylates (e.g. fruits and vegetables [75 - 78]) might excrete larger amounts of SA and its major metabolite, SU.

When urine from two groups of people who had not taken salicylate drugs (a group of strict vegetarians and a group of people with an unrestricted diet) was examined using the method developed, it was found that the group of vegetarians excreted significantly more ($p<0.01$) SU and SA (see Table VII). When Blacklock et al [88] determined the concentration of SA in the blood of people who took low-dose aspirin (75mg/day) regularly they found a median concentration of 10.03 μmol/l of SA and they noted that the range of concentrations of SA in this group (0.23 – 25.4μmol/l) was overlapped with the values recorded for the group of vegetarians (see Table VIII). In view of these findings it was decided to investigate the amounts of SA and SU excreted by people taking aspirin to see how these compared with those excreted by people with either a vegetarian diet or an unrestricted diet. Urine of two groups of people who took aspirin regularly (75mg/day or 150 mg/day) was examined for its content of salicylates. Approximately ten times as much SA and its metabolite was excreted by people taking aspirin (either 75mg/day or 150 mg/day) than by the vegetarians ($p < 0.0001$, Mann-Whitney U-tests, see Table VII). Complete hydrolysis of 75mg of acetylsalicylic acid yields 56mg of SA. Therefore, it appears that SA derived from consumption of aspirin tablets is more bioavailable than the SA within foodstuffs where it might be sequestered within a matrix of insoluble polymeric materials from which it must be released prior to absorption. However, the results of this

work show clearly that vegetarians are exposed to greater amounts of SA than people with an unrestricted diet.

Interestingly, urine from one person taking 75 mg of aspirin per day was devoid of SA and urine from another individual who had been taking 150mg of aspirin per day was devoid of SU (see Table VII). In the latter case, the urine contained a very high concentration of SA (over 64 μmol/l) and it is suggested that this individual might not have been capable of conjugating SA with glycine. This finding, along with the finding that people who had ingested aspirin (75 mg/day or 150 mg/day over a prolonged time period) excreted similar amounts of SA confirms the conclusion that the amount of SU excreted by an individual depends on the availability of glycine [112]. Hutt et al [113] reported that the extent to which man is capable of converting SA into its glycine and glucuronide conjugates is variable. They noted a 12-fold variation in the amount of SU excreted by 83 healthy volunteers. This variation between individuals in their ability to conjugate SA with glycine might explain the wide range of amounts of SA and SU excreted after receiving aspirin (see Table VII).

The complexity of the elimination of salicylates in man is well documented [112,113]. At low doses of aspirin, the rate of elimination of SU is first-order with respect to the dose of aspirin administered. However, this behaviour does not apply when larger doses are taken and when a smaller proportion of the dose is excreted as SU. Under these conditions increased concentrations of minor metabolites might be excreted [113] or, as found in this work, more is excreted as SA.

Miners et al [119] gave an oral dose of aspirin (900mg) to two groups of women and one group of men. Prior to the experiment none of the people had been taking aspirin for a period of at least a week. One of the groups of women had taken oral contraceptive steroids, the other group of women had not. Miners et al [119] observed that women who took contraceptive steroids excreted more SU than women who did not. They also found that men had a greater capacity to form the glycine conjugate (SU) than women (79% greater than in females) and concluded that the capacity to produce the glycine conjugate might be elevated in women who take contraceptive steroids. These results highlight two possible variables (gender and the possibility of induction by steroids) that might influence the extent of conjugation of SA.

The results shown in Table VII support the conclusions of Blacklock et al [88] and they indicate that there might be a correlation between the amount of salicylate excreted and the content of salicylates in food. However, information regarding the amount of salicylates in foods is difficult to interpret. In 1985 Swain et al [75] used diethyl ether to extract salicylates from foodstuffs and reported considerable amounts of "total salicylates" in herbs and spices. Their method employed UV quantification after HPLC with isocratic elution. However, their chromatographic procedure did not permit the complete separation of SA from other UV-absorbers.

Venema et al [76], unlike Swain et al [75], observed only small amounts of SA in foodstuffs; their values were similar to those reported by Variyar and Bandyopadhyay [77]. They also had used diethyl ether to extract the salicylates

from 30 different foods and then employed HPLC with isocratic elution followed by emission of fluorescence to determine concentrations of SA. They claimed that fluorescence was about 1000 times more sensitive than ultra-violet absorption for the determination of SA and concluded that this technique was more suitable for determining the very low amounts of SA that appeared to be present in herbs and spices.

Robertson and Kermode [78], Herrmann [96], Venema et al [76] and Variyar and Bandyopadhyay [77] all reported small amounts of salicylates in herbs and spices, their results being much lower than those reported by Swain et al [75]. It has been argued by Venema et al [76] that the considerably higher amounts that were found by Swain et al [75] were due to the incomplete separation of other UV-absorbing compounds.

Since the content of salicylates in foodstuffs is extremely controversial, it was decided to apply the method of Blacklock et al [88] to see if it might be suitable for the determination of salicylates in these materials. In this method the acidic and neutral hydrophobic compounds were extracted with ethyl acetate from acidified portions of homogenized soups, homogenized cooked foods, powdered spices and wines. The substances removed were separated using HPLC with a system of gradient elution and determined electrochemically. The repeatability of the method was determined using 4 different soups. The CV ranged from 0.7% to 6.8% (see Table XIII) and these are much lower than those reported by any of the previous investigators [76 - 78]. The intra-assay precision of the method (CV,

2.4%.) was calculated from values derived from 22 duplicate sets of determinations of SA in soups; this value also is lower than reported previously [79]. To assess the efficiency of the extraction stage of the method, paired portions of five different varieties of soup were examined. One of each pair received 15ng of SA per 1g of soup. The amounts of SA present in extracts of the soups were determined and it was estimated that 78%, 72%, 83%, 77% and 84% of the SA added to the five different soups was recovered. The better accuracy and reliability of the method of Blacklock et al [88] showed that it was more liable to give trustworthy results than those published previously.

The diet of strict vegetarians, consisting of a very high proportion of foods derived from plants, contains more SA than an unrestricted diet. Therefore, it was not surprising to find that blood and urine from a group of vegetarians contained more salicylates than blood and urine from people with an unrestricted diet (see Tables VII and VIII). Blood from a group of people living in a rural part of India contained 2.5 times more SA than blood from the vegetarians (see Table VIII) and this suggests that the Indians might have an even greater exposure to salicylates than the group of vegetarians. These Indians have, essentially, an organic vegetarian diet that is rich in herbs and spices and several of these spices have very high contents of salicylates (see Table XVI).

The amounts of "total" salicylates in four of the most commonly used spices in Indian cookery (chilli, cumin, paprika and turmeric) were appreciably higher than those of all of the other spices and foodstuffs examined, e.g. cumin contained

more than 1.5% by wt. From the values recorded in Table XVI, and knowing that spices such as chilli, cumin, turmeric and paprika are used in substantial amounts in Indian cookery, it is not unreasonable to conclude that the Indian population is exposed to dietary sources of salicylates to a much greater extent than Western populations. The results of this work show that there are higher concentrations of SA in the blood of a group of Indians and a group of people with a restricted vegetarian diet than in a group of people with an unrestricted diet (see Table VIII). SA is a secondary metabolite [13] and a defence hormone in plants [9] and, therefore, it is possible that food prepared from plants cultivated under "organic" conditions might contain more SA than food prepared from plants cultivated by "conventional" means. Organic conditions avoid the use of herbicides, fungicides and pesticides. Plants cultivated under these conditions, therefore, might have higher concentrations of substances such as SA that are known to be involved in initiating the systemic acquired resistance response [9-16]. Many of the ingredients used to prepare the foods eaten by the Indians and vegetarians had been derived from plants that were cultivated under organic conditions and they might contain more SA than plants cultivated under "conventional" conditions. To test the hypothesis that foods prepared from ingredients derived from plants that had been cultivated under organic conditions might contain more SA than foods prepared from conventionally-produced ingredients, the content of SA in 17 wines and in 35 vegetable soups were determined. When wines were investigated, the amount of SA found in organic wines appeared to be greater than that in wines prepared from conventionally-grown grapes. However, there was an overlap in the range of values observed and only a few wines of each type were examined (see

Table XV). The amounts of SA in late-harvested wine and in wine that had been produced from grapes infected with *Botrytis* were appreciably higher than the amounts in the other wines; however, only a few wines of each type were examined (see Table XV).

When soups were examined, SA was a component of all of the "organic soups" and most (n = 20) of the "conventional" soups. SA did not appear to be present in 4 of the "conventional" soups. The amount of SA found in soups that had been prepared from "organically grown" ingredients was significantly higher ($p = 0.0032$) than the amount in the soups prepared from "conventionally-grown" ingredients (see Table XIV).

It is concluded that foods prepared from plant materials, especially those derived from plants grown organically, contained appreciable amounts of SA (see Tables XIV - XVII). It is concluded also that the group of vegetarians had an increased dietary exposure to salicylates. However, their extent of exposure probably was lower than that of the rural Indians (see Tables VII and VIII). To find out if the salicylates of such plant-derived ingredients survived the harsh conditions of cooking, the content of salicylates in four cooked Indian-style dishes were determined. Salicylates were found in all four dishes; higher concentrations were present in the freshly prepared cooked foods obtained from the Indian restaurant (see Table XVII).

Large amounts of salicylates were present in traditional Indian dishes and higher amounts were found in foods prepared from ingredients cultivated under "organic" conditions. If these salicylates were bioavailable their high contents in the foods consumed might explain the high concentrations of SA in the blood of the group of Indians and the low incidence of colorectal cancer among these people. Rural India has the lowest incidence of colorectal cancer in the world [86]. To find out if salicylates within such cooked dishes were bioavailable, the concentration of SA in blood and SA and SU excreted in the urine of an individual who had fasted for 10h prior to eating vegetable Vindaloo containing 94.03mg of salicylates were determined at various times throughout the subsequent 7h period. A three-fold increase in the concentration of SA in blood was observed within 2h of consuming the food. A five-fold increase in the rate of excretion of SA and SU was noted 2h after that (see Fig 20). From these results it is concluded that absorption of the salicylates from food occurred within 2h and that metabolism of SA occurred rapidly thereafter (see Fig. 20). From the amount of total salicylates consumed and the amount of SA and SU excreted in urine, it was calculated that at least 3% of the salicylates ingested were bioavailable. In this context it is worthy of mention that previous investigators of the bioavailability of low molecular dietary components (such as flavanoids) from food have reported values of less than 1% [120].

It has been established that prolonged consumption of aspirin exerts a chemoprotective effect against colorectal cancer [126] and the body of evidence supporting this observation is enormous [115,123,124]. Some of the evidence has

emerged from epidemiological studies. Many of these are either large case-controlled (retrospective) or large cohort (prospective) investigations. They reveal significant decreases of 30% - 50% in the incidence of colorectal cancer [60, 115, 116, 122, 123]. Baron et al [122], Sandler et al [57] and Benamourzig et al [133] showed that taking aspirin exerts chemoprotection against the development of adenomas.

Several researchers have investigated the dose of aspirin required to afford protection and Giovannucci [124] has concluded that low doses of aspirin (81 mg per day) taken over one or two decades are sufficient. Baron et al [122] found that the greatest extent of chemoprotection was at a dose of 81mg and the results presented here show that certain cooked foods have similar contents of salicylates (see Table XVII). The results of Tables VII and VIII show that vegetarians have concentrations of SA in blood and of SA and SU in urine that overlap with concentrations of these compounds measured in blood and urine from a group of people who had regularly been taking aspirin. Therefore it is possible that the benefits to health of a vegetarian diet are due to an increased exposure to dietary salicylates.

The findings of this work show that the food consumed by people in India contains large amounts of salicylates and that >3% of SA derived from typical meals may become bioavailable. The daily intake of salicylates of this population may even be larger than that of people who take low-dose aspirin. A low dose of aspirin (81mg) can generate a maximum of 60.5 mg of SA, of which about 60% is

bioavailable) [97]. From these observations it is tempting to speculate that the low incidence of colorectal cancer within the Indian population is due to their high exposure to dietary salicylates throughout their lifetime.

The chemoprotective effects of taking aspirin against a variety of conditions such as cardiovascular disease, stroke and various soft tissue cancers [45 – 65, 127] have been reported. Recently, Shtivelband et al [135] found that aspirin and sodium salicylate suppressed the production of cancer-induced COX-2 proteins at pharmacological (millimolar) concentrations. Aspirin is an unstable phenolic ester and it is very difficult to prepare an aqueous solution of aspirin with which to dose either animals or cultured cells. Aspirin is hydrolysed rapidly to give SA even on exposure to moist air and it decomposes in aqueous solutions most slowly at about pH 2.5. Most investigators of the mechanisms of the chemoprotective effects of aspirin do not appear to be aware of its instability and they, at best, probably recorded the effects of mixtures of aspirin and SA, with SA being the predominant component [66 – 71]. Other workers [29] have tried to minimize the extent of hydrolysis. However, they were forced to increase the pH of their acidic "solutions of aspirin" prior to adding them to culture media. Therefore, it is not unreasonable to propose that SA, generated by the hydrolytic decomposition of aspirin, produced the chemoprotective effects noted by these investigators.

The evidence presented in this work suggests strongly that dietary salicylates generated SA *in vivo* and it is this substance that affords protection against colorectal cancer. Preliminary results suggested that the dietary sources of SA

were bioavailable (see Fig. 19). It would appear, however, that another source(s) maintained a steady rate of excretion of salicylates in the absence of a dietary source (see Fig. 21). In order to eliminate any dietary source of SA, an individual consumed milk and water for three days; SA and SU were not present in the milk and the water. Throughout the time-course of this experiment the individual was isolated from dietary SA and any SA derived from earlier dietary sources should have been cleared from the blood and urine within 72h (SA in serum has a half-life of 2-4h). These findings show that under these conditions a rate of excretion of salicylates of 2.1 μmol/24h was maintained. The results confirm the view that a source(s), other than dietary salicylates, maintained the steady slow rate of excretion (see Figs. 19 and 21).

Armstrong et al [24] suggested that the metabolic activities of gut bacteria or the endogenous transformation of dietary precursors might contribute to the salicylates found in people who had not taken salicylate drugs. To investigate the possible contribution by gut microflora, the concentration of SA in serum from "germ-free" rats (without gut microflora) was compared with that found in serum from ordinary rats. The foodstuffs that had been fed to both of these groups of animals did not contain SA. When sera from both groups were examined it was found that sera from the "germ-free" animals contained concentrations of SA that were about 2.5 times higher than sera from the conventionally-reared animals. There is no obvious explanation as to the cause of the higher concentration of SA in the blood of "germ-free" rats. However, the presence of SA in the blood of both groups of animals supports the conclusion that a source(s) other than dietary

salicylates and the metabolic activity of gut bacteria had contributed to the concentration of SA in the blood of the "germ-free" rats.

Chorismic acid, *o*-coumaric acid and benzoic acid are precursors of SA in plants and some bacteria [4 - 8]. Chorismic acid and *o*-coumaric acid are intermediates of the shikimic acid pathway and phenylpropanoid pathway respectively (see Figs. 1 and 2). When serum from rats was examined to see if it contained chorismic acid or *o*-coumaric acid, the results obtained suggested that chorismic acid might have been present (see Table XIX). Benzoic acid is widely used as a food preservative [129] and it was possible that the people studied might have ingested this substance (especially if their diet contained processed and preserved foodstuffs). The group of non-vegetarians that were investigated in this work and by Blacklock et al [88] had an unrestricted diet that probably contained foodstuffs that contained sodium benzoate (see Tables VII and VIII).

To find out if a dietary source of benzoic acid affected the concentration of SA in blood and the amounts of SA and SU excreted, sodium benzoate was administered orally to two male volunteers who had not taken salicylate drugs for a period of 2 weeks prior to the investigation. Both consumed food from the same batches at the same time and in identical quantities over a period of 4 days (see Materials and Methods 2.2.3a). On the third and fourth days of this dietary scheme one volunteer took 2.0g of sodium benzoate and the other took 1.0g. The serum and urine from these individuals were examined and it was found that, for both individuals, the rates of excretion of SA and SU were increased (see Fig. 22) on the day after sodium benzoate was first consumed. These results indicate that

benzoic acid might have been transformed into SA *in vivo* and that dietary benzoic acid might have contributed to the steady rate of excretion of salicylates observed in these investigations (see Figs 19 and 22) and contributed to the SA concentrations measured in serum from people who had taken salicylate drugs (see Table VIII).

Prior to excretion, benzoic acid becomes conjugated with glycine to form hippuric acid, and the extent of its conjugation depends also on the amount of glycine available [130]. In man, benzoic acid arises primarily from dietary sources and from aromatic amino acids by the metabolic activity of gut microflora [131,132,23]. Asatoor [23] reported that intestinal bacteria might also synthesize benzoic acid from non-aromatic precursors. When Brewster et al [22] investigated the metabolism of shikimate in rats, they found that the production of hippuric acid resulted from an initial reduction and dehydroxylation of shikimic acid (to form benzoic acid). However, they could only speculate that the transformation of benzoic acid into other aromatic compounds such as SA might have occurred in mammalian tissues.

In order to see whether or not non-human blood contained SA, the blood from a wide variety of animals with very different dietary habits was examined to see if it contained SA and at what concentrations. SA (range, 0.033–9.854 μmol/l; see Table XVIII) was present in blood from mammals, birds, fish and reptiles. However, since reliable dietary information concerning the animals was not available it is inappropriate to speculate about these values and to compare them with concentrations of SA found in blood from humans (see Table VIII).

However, it is interesting to note that SA was present in the blood of the vast majority of these animals.

The present work has revealed that that there are several possible sources that might account for the presence of salicylates in the blood and urine of people who had not taken salicylate drugs. Dietary salicylates and dietary precursors of SA, such as benzoic acid and chorismic acid, might be responsible for maintaining almost steady rates of excretion of SA and SU in the urine of people who had not taken salicylate drugs. The slopes of the linear fits illustrated in Fig. 19 and 21 indicate that, during fasting and when food that does not contain salicylates is consumed, there is a steady rate of excretion of salicylates (SA plus SU) [range, 2.1 – 10.9 μmol/24h]. These results suggest that, in addition to dietary salicylates, there are two other possible sources of the salicylates found in the blood and urine of people who had not taken salicylate drugs; an endogenous source and dietary precursors of SA that are transformed in vivo.

Janssen et al [80] showed that there was a positive correlation between diets that were rich in plant-based foodstuffs and the amount of salicylates excreted in urine. This work supports finding of Janssen et al [80]. Moreover, it shows that foodstuffs, such as soups, wines and vegetarian Indian dishes (all prepared from plant materials) contained appreciable amounts of SA and that the salicylates in cooked foods were bioavailable. Blacklock et al [88] found higher concentrations of SA in the blood of a group of vegetarians than in the blood of a group of people with an unrestricted diet. This work extends this finding; vegetarians excreted

almost three times more SA and SU in their urine than people with an unrestricted diet (see Table VII). These results suggest that the major source of the salicylates in people who do not take salicylate drugs are the salicylates present in food.

4.1 Conclusions and further work

a. Conclusions

It has been shown conclusively that SA and SU are constituents of the urine of people who had not taken salicylate drugs. Evidence as to the identity of these two compounds was gathered from enzymic and chromatographic studies and the identification was confirmed by using GCMS.

The lack of a reliable method for determining the concentrations of SA and SU in the urine of people who had not taken salicylate drugs prompted the development of a sensitive, accurate and precise analytical method. When the method of Blacklock et al [88], which had been developed for determining the concentration of SA in blood, was investigated it was found that it could be used with confidence to determine the content of SA in foodstuffs. These methods were used to assess the extent of people's exposure to salicylates by measuring the content of salicylates in foodstuffs, of SA in blood and urine, and of SU in urine.

The content of salicylates in certain foodstuffs prepared from ingredients derived from plants cultivated under organic conditions was significantly higher than similar foodstuffs prepared from ingredients cultivated conventionally. Large amounts of salicylates were present in spices. These findings, in conjunction with the finding that salicylates in cooked foods were bioavailable suggested that diet influences strongly the extent of exposure to salicylates.

Published epidemiological studies have indicated that a diet rich in fruits and vegetables is beneficial to health. Larger amounts of salicylates were found in the urine of a group of vegetarians than in a group of people with an unrestricted diet and it was concluded that the vegetarians had a greater exposure to salicylates. However, blood and urine from people who had been taking aspirin regularly contained even greater amounts of SA and SU.

People who have taken aspirin over a long period of time (2 or 3 decades) are afforded protection against a variety of cancers. The results of this work indicate that these protective effects might be due to a long-term exposure to SA, which is generated rapidly from the aspirin consumed.

High concentrations of SA were present in blood from a group of Indian people living in a rural area. These people had eaten foods that were prepared from ingredients that were cultivated under organic conditions and they contained large amounts of spices. This investigation has provided evidence that people who are predominately vegetarian, and who eat foods prepared according to traditional Indian recipes are exposed to appreciable amounts of SA throughout their lifetime. It is proposed that this extensive and chronic exposure to salicylates might account for their greatly reduced susceptibility to colorectal cancer.

The results presented have shown clearly that a source(s) of SA other than dietary salicylates maintain(s) a steady rate of excretion of salicylates in man when dietary salicylates are excluded.

It is possible that precursors of SA might be present in foodstuffs. Chromatographic evidence suggested that one of these precursors, chorismic acid, might be present in serum. Increased rates of excretion of SA and SU were observed after two people ingested another possible precursor, benzoic acid. These results indicate that dietary benzoic acid, and possibly chorismic acid, also might be absorbed from foodstuffs and transformed into SA.

The possible contribution of gut bacteria to the amounts of salicylates found in blood was investigated by examining conventionally-reared and "germ-free" rats. Blood from the "germ-free" animals contained higher concentrations of SA than blood from conventionally-reared animals. These findings confirm that a source(s) other than dietary SA and the metabolism of gut bacteria are responsible for the presence of SA in serum.

b. Further work

A more thorough assessment of the extent of man's exposure to salicylates would need determinations of the amount of "total" salicylates in blood, urine and foodstuffs. It is suggested that these materials should be treated with alkali prior to determination of SA (see 2.3.4).

Further experiments to investigate the presence of precursors of SA, such as chorismic acid, in foodstuffs are required. Initially, the identity of the substance with the chromatographic characteristics of chorismic acid, which was present in blood, should be established. Thereafter, its presence in foodstuffs and urine should be investigated; its quantification would enable investigations to be made

of the possible contribution of chorismic acid to salicylates found in man. Such experiments might allow us to see if chorismic acid is a constituent of the diet, is bioavailable, and can be transformed into SA in man.

Armstrong et al [136] devised experiments to investigate the origin of urinary hippuric acid by using radio-labelled precursors. The use of ^{14}C-labelled benzoic and chorismic acids is suggested for further work. After ingestion, the extent of incorporation of ^{14}C from these compounds into the salicylates in blood and urine would reveal if they were transformed significantly *in vivo*.

An investigation of the possible contribution of "environmental" salicylates, such as the salicylate esters added to toothpastes and mouthwashes, also should be made in an attempt to assess more fully the exposure to salicylates.

Finally, a comparison of the long-term exposure of man to aspirin with the long-term exposure to SA should be made to investigate the idea that chemoprotection against colorectal cancers afforded by consumption of aspirin is caused by SA. Such an investigation would be difficult or impossible to arrange; large numbers of volunteers would be required and ethical permission would, almost certainly, be withheld. Therefore, it might be more realistic to employ laboratory animals in these investigations. A typical experiment might use three groups of animals. One group would be given doses of aspirin, a second group doses of SA, the third untreated group serving as the control. The three groups of animals then would be exposed to a known inducer of colorectal cancer.

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