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Effects of salicylic and acetylsalicylic acid alone and in combination on platelet aggregation and prostanoid synthesis in man

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1 The present study was designed to investigate the effects of salicylate on the anti-platelet action of acetylsalicylic acid as well as on *in vivo* prostanoid formation and platelet function in healthy volunteers.

2 In the first study six female volunteers received 350 mg acetylsalicylic acid intravenously, with and without previous oral administration of sodium salicylate (1200 mg daily for 3 days). Urinary prostanoid excretion as well as platelet aggregation and thromboxane formation were measured before and during salicylate and after acetylsalicylic acid. In the second study seven female volunteers received sodium salicylate (52.6 mg kg^{-1}) or acetylsalicylic acid (60.7 mg kg^{-1}) for 8 days in a randomized cross-over protocol. Urinary prostanoid excretion, platelet aggregation and thromboxane formation as well as salicylate plasma concentrations were determined before, during and after administration of each drug.

3 Sodium salicylate did not impair the complete suppression of arachidonic acid-induced platelet thromboxane formation and aggregation obtained by the single intravenous dose of acetylsalicylic acid in the first study.

4 Sodium salicylate in the second study did not affect urinary excretion of prostaglandin E_2, its major urinary metabolite (7α-hydroxy-5,11-diketo-tetranor-prostane-1,16-dioic acid), and 2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$, the main urinary metabolite of epoprostenol (prostacyclin, PGI_2). In contrast, acetylsalicylic acid significantly decreased excretion rates of these prostanoids by 64, 59 and 61%, respectively.

5 In both studies platelet aggregation and thromboxane formation induced by collagen, thrombin or arachidonic acid were not significantly affected by salicylate administration, whereas acetylsalicylic acid inhibited platelet aggregation induced by all three agents as well as thrombin- and arachidonic acid induced thromboxane formation.

6 In conclusion, salicylate *in vivo* does not affect the anti-platelet efficacy of acetylsalicylic acid in man. Furthermore, salicylate in a dose known to be effective in the symptomatic treatment of rheumatic diseases—in contrast to the acetylated drug—does not affect prostanoid formation.

Keywords acetylsalicylic acid sodium salicylate prostanoids blood platelets thrombosis

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Introduction

While the inhibitory effect of acetylsalicylic acid on platelet function (O'Brien, 1968; Weiss *et al.*, 1968; Zucker & Peterson, 1970) and prostanoid formation (Smith & Willis, 1971; Vane, 1971) is well known (Atkinson & Collier, 1980), the effects of sodium salicylate are much less defined. Although salicylate has been shown to be similarly potent as an analgesic and anti-inflammatory drug (Atkinson & Collier, 1980; Rainsford, 1984), it has almost no effect on prostanoid formation of gastric mucosa (Ligumsky *et al.*, 1982; Whittle *et al.*, 1980), macrophages (Kuehl *et al.*, 1980), ram seminal vesicle microsomes (Humes *et al.*, 1981), or thrombocytes (Roberts *et al.*, 1984; Smith & Willis, 1971; Vargaftig, 1978a). Platelet function is not affected by salicylate as well (O'Brien, 1968; Roberts *et al.*, 1984; Smith & Willis, 1971; Weiss *et al.*, 1968; Zucker & Peterson, 1970). In contrast prostaglandin formation in inflamed tissue exudate is decreased by the drug (Higgs *et al.*, 1976; Smith *et al.*, 1979; Whittle *et al.*, 1980) and oral application of 3 g sodium salicylate per day has been shown to reduce urinary excretion of the main prostaglandin E metabolite (Hamberg, 1972), an observation which has led to the investigative use of sodium salicylate as an inhibitor of endogenous prostaglandin production (Robertson & Chen, 1977).

Furthermore, it has been suggested that sodium salicylate may decrease the effect of other non-steroidal anti-inflamatory drugs on cyclooxygenase activity. Thus, inhibition of ram seminal vesicle microsomal cyclooxygenase by acetylsalicylic acid was diminished by addition of salicylate *in vitro* (Humes *et al.*, 1981). *In vivo* the amount of gastric ulcerations induced by acetylsalicylic acid, indomethacin or other inhibitors of prostaglandin synthesis was decreased by simultaneous administration of sodium salicylate in the rat (Ezer *et al.*, 1976). In several *in vivo* and *in vitro* experiments performed in rabbits or rats sodium salicylate interfered with the inhibition of platelet function and/or platelet prostanoid synthesis caused by acetylsalicylic acid (Buchanan & Hirsh, 1984; Dejana *et al.*, 1981; Vargaftig, 1978a,b.) *In vitro* studies using human platelet-rich plasma provided evidence that salicylate might diminish the effect of acetylsalicylic acid on cyclooxygenase and platelet function (Ali & McDonald, 1979; Brantmark *et al.*, 1981; Dahl *et al.*, 1983). Because salicylate is formed rapidly *in vivo* after administration of acetylsalicylic acid and then accumulates during prolonged treatment (Levy & Tsuchiya, 1972), this interference has been proposed to be of importance during antithrombotic prophylaxis with acetylsalicylic acid (Castellarnau *et al.*, 1981; Dejana *et al.*, 1983). However, *in vivo* thrombus formation in the rat was found to be diminished after administration of acetylsalicylic acid (10 mg kg^{-1}) irrespective of pretreatment with sodium salicylate in different doses thus suggesting no effect of salicylate on the antithrombotic action of the acetylated compound (Philp & Paul, 1981). When acetylsalicylic acid was administered at doses between 20 and 1500 mg daily, thromboxane B_2 (TXB_2) formation in serum remained suppressed during a prolonged treatment period up to 30 days (Patrignani *et al.*, 1982; Viinikka *et al.*, 1983; Weksler *et al.*, 1983), and administration of sodium salicylate did not affect the inhibitory effect of acetylsalicylic acid on serum thromboxane formation and platelet aggregation (Dahl & Uotila, 1984; Nitelius *et al.*, 1984). These studies make a clinically relevant interaction between these two drugs less likely.

In order to investigate further the effect of each drug separately as well as in combination on platelet aggregation and *in vivo* prostaglandin formation, we have performed the following experiments in normal volunteers.

Methods

Study design

The studies, which have been approved by the Ethics Committee of the Robert-Bosch-Hospital, Stuttgart, were performed in healthy volunteers, who had given their written informed consent. Female subjects were chosen because contamination of urine with seminal fluid can give erroneous results for prostanoid production rates in males (Patrono *et al.*, 1979).

Interaction between sodium salicylate and acetylsalicylic acid

The study, which was performed in six volunteers (age 26 ± 1 years, body weight 64.0 ± 4.3 kg), consisted of two parts. In the first part a single intravenous dose of acetylsalicylic acid was given after sodium salicylate had been administered for 3 days. In the second part acetylsalicylic acid was given again as a single intravenous dose following 2 days of placebo administration. The sequence of the two parts of the study was randomized with a minimum 2 week wash-out period between them.

Each volunteer received sodium salicylate at a dose of 1200 mg daily in two divided doses for 3 days. On the morning of the fourth day 350 mg acetylsalicylic acid was administered intra-

venously 2 h after the last dose of sodium salicylate. Blood samples were collected for platelet function studies before and during salicylate administration as well as 1 h, 46 h and 1 week after acetylsalicylic acid. A blood sample for determination of the salicylate plasma level was obtained before the last dose of sodium salicylate, i.e. 12 h after the previous drug intake. Urine was collected before, during and after the salicylate period for one 24 h period.

In the second part of the study the same volunteers received placebo for 2 days. Again, on the next day 350 mg acetylsalicylic acid was administered intravenously 2 h after the last placebo. Blood and urine were collected before and after the drug in the same intervals as in the other part of the study.

Comparison between the effects of salicylic and acetylsalicylic acid

This study, which was performed in seven volunteers (age 24 ± 2 years, body weight 62.5 ± 5.1 kg), again was divided into two parts in which either sodium salicylate or acetylsalicylic acid were given for 8 days. The sequence of both drugs was randomized with a wash-out period of 19 days.

After a 2 day control period the volunteers received sodium salicylate (mean dosage for all seven volunteers 52.6 ± 2.3 mg kg^{-1} daily) or acetylsalicylic acid (60.7 ± 2.7 mg kg^{-1} daily) divided into three doses (08.30, 15.00 and 21.30 h) for 8 days. A final control period followed after another 17 days. Urines (22 h) were collected on the last 2 days of each of the five experimental periods. Blood for determination of salicylate plasma levels was sampled on the last 3 days of each drug period at 15.00 h i.e. 6.5 h after the previous administration. Platelet function studies were performed on the last 2 days of each drug period at 15.00 h, on the last two mornings before each drug period, and on the last 2 days of the final control period at 08.30 h each.

Drugs

Gelatine capsules were prepared with sodium salicylate (295 mg $capsule^{-1}$) or acetylsalicylic acid (340 mg $capsule^{-1}$) obtained as powders from Chemische Fabrik Aubing (München, F.R.G.) and Bayer AG (Leverkusen, F.R.G.), respectively. Identical capsules filled with lactose served as placebo. For intravenous administration (±)-lysine-mono-acetylsalicylate (Aspisol®, Bayer AG, Leverkusen, F.R.G.) was used.

Salicylate plasma concentrations

Salicylate plasma concentrations were determined similarly as described (Buskin *et al.*, 1982; Caterson *et al.*, 1978). In brief, 200 to 500 μl plasma were spiked with an internal standard (5-propionyl-aminosalicylic acid) (Fischer *et al.*, 1984), acidified with 100 μl phosphoric acid (1M), vortexed and extracted into 2 ml diethylether. After evaporation in an ice-bath, the material was applied to a high-pressure liquid chromatography column (5 μm Bondapak C18, Waters Assoc.), eluted with water/methanol/acetic acid (77/22/1), and peaks were detected by U.V. absorption (237 nm).

Sodium, potassium and creatinine in urine

Sodium and potassium were determined by flame photometry, and creatinine concentration by use of a modified Jaffe's reaction.

Urinary prostanoid excretion

Urinary excretion of 7α-hydroxy-5,11-diketo-tetranor-prostane-1,16-dioic acid, the major urinary metabolite of PGE_1 and PGE_2 (PGE-M), was determined similarly as described (Frölich, 1977; Müller *et al.*, 1981). An aliquot of the urine was spiked with [^{3}H]-PGE-M, and purified by reversed phase silica cartridge (SepPak C18, Waters Assoc.) and h.p.l.c. Quantitative analysis was performed by gas chromatography mass spectrometry (GC-MS).

For determination of 2,3-dinor-6-keto-$PGF_{1\alpha}$ by GC-MS urine was added with tritiated and deuterated 2,3-dinor-6-keto-$PGF_{1\alpha}$ which were prepared from 6-keto-$PGF_{1\alpha}$ by bacterial β-oxidation (Falardeau *et al.*, 1981). [^{3}H]-6-keto-$PGF_{1\alpha}$ was obtained from New England Nuclear (Dreieich, FRG), whereas 8,9,10,10-$^2H_4$-6-keto-$PGF_{1\alpha}$ was synthesized chemically (Meese, 1983). Purification of urine samples were performed by extraction, SepPak C18 and h.p.l.c.

Prostaglandin E_2 was determined by radioimmunoassay (RIA) after purification of the urine by open silicic acid column chromatography (Dray *et al.*, 1975). [^{3}H]-PGE_2 and the PGE_2 antibody were purchased from New England Nuclear (Dreieich, FRG) and the Institut Pasteur (Paris, France), respectively.

Platelet function and eicosanoid formation

Blood (9 ml) was collected in a syringe containing 1 ml of 3.13% sodium citrate. Platelet-rich plasma (PRP) was generated by centrifugation (200 *g*, 15 min). The pellet was then recentrifuged

to give platelet-poor plasma which was used to adjust the platelet-count of the PRP to 200 000 μl^{-1}. Platelets were counted in an automatic counter (ELT-8, Dr. Molter). Aggregation was assessed by a turbidimetric method (Labor-Aggregometer) as described (Born, 1962). For arachidonic acid-induced aggregation the substrate (NuChek Prep., Elysian, USA) was dissolved in 0.1 M Na_2CO_3 (20 mg ml^{-1}), added to 0.3 ml PRP at different concentrations between 0.11 and 1.71 mM, and the lag time until 10% increase of light transmission in the aggregometer occurred was determined ($t_{10\%}$). Three minutes after addition of arachidonic acid the reaction was quenched with 0.5 ml ethanol. After centrifugation the solution was diluted with phosphate buffer and then analyzed by RIA for TXB_2.[^{3}H]-TXB_2 was supplied by New England Nuclear, unlabeled TXB_2 was kindly provided by U.Axen (The Upjohn Co., Kalamazoo, USA). The TXB_2 antibody was generated by J. B. Smith (Lewy *et al.*, 1975).

During the second part of the study aggregation was induced by collagen (Kollagen-Reagens Horm®, Hormon-Chemie, München) (2 μg ml^{-1} PRP) and thrombin (Test-Thrombin®, Behring Institut, Marburg) (0.25 u ml^{-1} PRP), respectively. The samples (1 ml PRP) were stirred for 3 min after addition of the stimulus and then incubated at 37°C for 30 min. For determination of TXB_2 0.1 ml PRP was then added to 1 ml ice-cold ethanol, centrifuged, diluted with buffer, and analysed by RIA.

Statistical analysis

The results are expressed as means and s.d. unless stated differently. The statistical significance of differences between treatment groups was assessed by use of Wilcoxon's signed rank test; if appropriate, Friedman's test was used for evaluation of differences between several treatment groups. A difference was considered as significant when $P < 0.05$.

Results

Interaction between sodium salicylate and acetylsalicylic acid

Salicylate plasma concentration During administration of sodium salicylate trough salicylate plasma levels were 0.16 ± 0.15 mM before the last dose of sodium salicylate.

Urinary prostanoid excretion Urinary excretion of PGE_2 and PGE-M did not change significantly during administration of salicylate or after a single intravenous dose of acetylsalicylic acid (350 mg) when measured in a 24 h urine sample (Table 1).

Table 1 Urinary excretion of PGE_2 and PGE-M during administration of sodium salicylate (1200 mg day^{-1}) (SA) and after a single intravenous dose of acetylsalicylic acid (ASA), both with and without concomitant oral administration of salicylate. The mean of the four control periods which did not differ significantly between one another is given as 'Control'.

	PGE_2 (ng day^{-1})	*PGE-M (ng day^{-1})*
Control	105 ± 65	18,120 ± 8,810
ASA	85 ± 54	13,660 ± 5,948
SA	111 ± 56	16,560 ± 12,813
SA + ASA	94 ± 51	15,270 ± 16,659

Platelet function and eicosanoid formation Arachidonic acid-induced platelet aggregation and TXB_2 synthesis are shown in Figure 1. Acetylsalicylic acid decreased thromboxane formation to 1–2% of the basal value already 1 h after its intravenous administration; this effect remained unchanged until 46 h after injection. Concomitantly platelet aggregation was completely abolished throughout this period (not shown in Figure 1). After 3 days of oral administration of 1200 mg sodium salicylate neither platelet aggregation nor platelet thromboxane formation were significantly altered. Furthermore, administration of acetylsalicylic acid had the same inhibitory effect on platelet function as observed without salicylate pretreatment. Seven days after administration of acetylsalicylic acid platelet aggregation was completely restored in both experimental periods, whereas thromboxane formation still tended to be somewhat inhibited; this difference, however, was only significant for the highest concentration of arachidonic acid in the salicylate pretreated group.

Comparison between the effects of salicylate and acetylsalicylic acid

Salicylate plasma concentration Salicylate concentrations on the last 3 days of each treatment period did not differ from one another significantly thus showing that steady state had been achieved. On the last day the plasma levels were 1.18 ± 0.44 mM after acetylsalicylic acid and 1.05 ± 0.24 mM after sodium salicylate ($P < 0.05$) when determined 6.5 h after the previous drug intake.

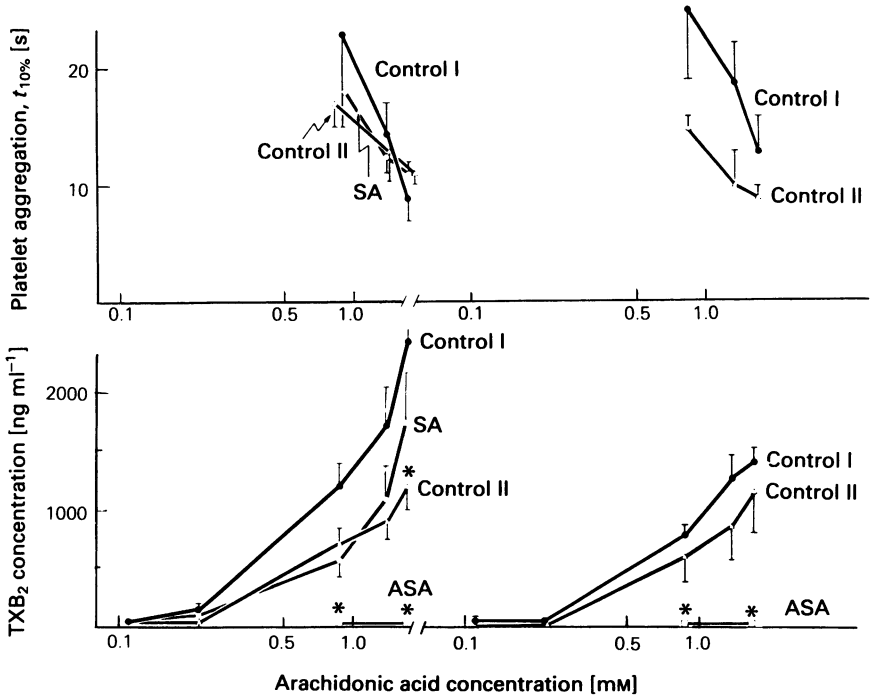

Figure 1 Dose-response curve of arachidonic acid on platelet aggregation (lag time to 10% increase of light transmission, $t_{10\%}$) and thromboxane formation (TXB_2). Left, before salicylate administration (Control I), on the fourth day of salicylate administration (SA), 1 h after intravenous application of acetylsalicylic acid (ASA), and 7 days later (Control II). Right, same experiment, during administration of placebo of salicylate. Platelet aggregation was completely inhibited by ASA in each case (not shown). Mean ± s.e. mean results, $n = 6$. * $P \leq 0.05$ *vs* control I, Wilcoxon test.

Urine flow and electrolyte excretion Urine volumes (22 h collection periods) were 1244 ± 513 ml during salicylate and 1044 ± 312 ml during acetylsalicylic acid which did not differ significantly from the means of their adjacent control periods (978 ± 466 and 934 ± 450 ml, respectively). Urinary excretion rates of sodium (mean of the three control periods 100.3 ± 29.2 mEq g^{-1} creatinine) and potassium (44.5 ± 12.5 mEq g^{-1} creatinine) did not change significantly during administration of either drug.

Urinary prostanoid excretion Urinary prostanoid excretion for each study period is given in Figure 2. Because the values of the three control periods did not differ significantly from one another, their means are presented. Excretion rates of PGE_2, PGE-M, and of 2,3-dinor-6-keto-$PGF_{1\alpha}$ were significantly reduced to 36.2, 41.0, and 38.6% of their control values during administration of acetylsalicylic acid. In contrast, sodium salicylate had no effect on urinary excretion of any of these prostanoids. The same results were obtained when the measured excretion rates were correlated to time instead to creatinine excretion.

Platelet function and eicosanoid formation Platelet thromboxane formation caused by collagen (2 μg ml^{-1} PRP) and thrombin (0.25 u ml^{-1} PRP) was decreased by acetylsalicylic acid to 4.0% and 7.0% of the control values (Figure 3). The rate of collagen-induced aggregation concomitantly decreased from 1.48 to 0.88 % s^{-1} ($P < 0.05$). Thrombin-induced aggregation was not impaired by the drug. Sodium salicylate in contrast did not affect platelet aggregation or thromboxane formation.

Discussion

In our present study we have compared the effects of acetylsalicylic acid and salicylate, its pharmacologically active metabolite, on platelet function and prostanoid formation in man and investigated the influence of salicylate on the anti-platelet effects of the parent compound. Like in most previous studies (O'Brien, 1968; Roberts *et al.*, 1984; Smith & Willis, 1971; Vargaftig, 1978a; Weiss *et al.*, 1968; Zucker & Peterson, 1970) administration of sodium salicylate (18.8 mg kg^{-1} for 3 days) did not affect

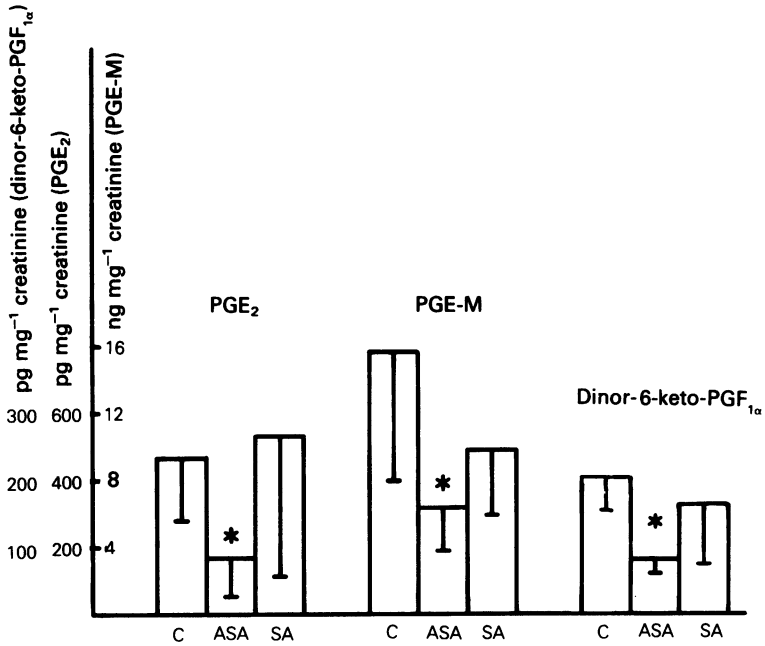

Figure 2 Effect of acetylsalicylic acid (ASA) and sodium salicylate (SA) on urinary excretion rates of prostaglandin E_2 (PGE_2), 7α-hydroxy-5,11-diketo-tetranor-prostane-1,16-dioic acid (PGE-M), and 2,3-dinor-6-keto-$PGF_{1\alpha}$. Mean ± s.d., $n = 7$ * $P < 0.05$, Wilcoxon test.

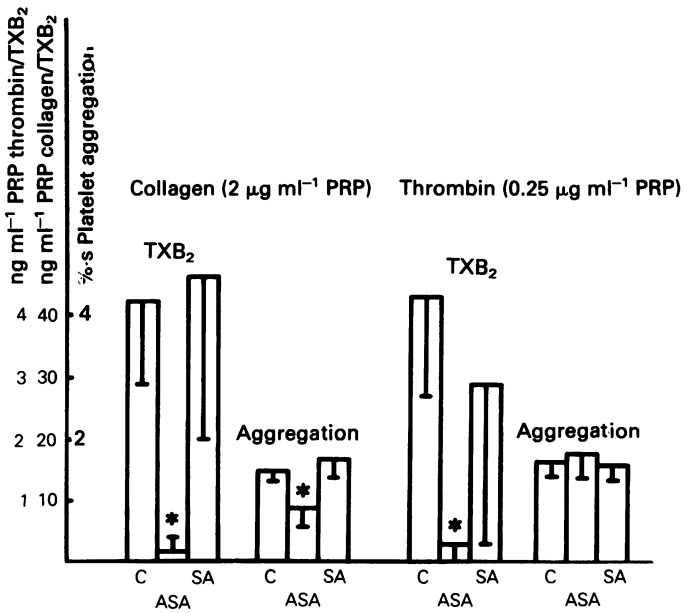

Figure 3 Effect of acetylsalicylic acid (ASA) and sodium salicylate (SA) on platelet aggregation (maximum change of light transmission, % s^{-1}) and thromboxane formation (TXB_2) induced by collagen or thrombin. Mean ± s.d., $n = 7$. * $P < 0.05$, Wilcoxon test.

platelet aggregation or thromboxane formation induced by arachidonic acid. Furthermore, when 350 mg acetylsalicylic acid was administered intravenously after pretreatment with salicylate, arachidonic acid-induced platelet aggregation and thromboxane formation were

inhibited completely. This result is in contrast to animal experiments using high doses of salicylate or *in vitro* studies with human platelets (Brantmark *et al.*, 1981; Dahl *et al.*, 1983) in which salicylate blocked the anti-platelet effect of acetylsalicylic acid. On the other hand, however, pretreatment with a single oral dose of salicylate (Dahl & Uotila, 1984) as well as prolonged administration of acetylsalicylic acid (Patrignani *et al.*, 1982; Viinikka *et al.*, 1983; Weksler *et al.*, 1983) which leads to cumulation of salicylate in plasma, did not interfere with the inhibitory efficacy of acetylsalicylic acid on platelet function in man. In our study the dose of salicylate has been chosen equal to the aspirin dose used in the myocardial reinfarction trials (Circulation, 1980). As the dose of acetylsalicylic acid was supramaximal with respect to inhibition of platelet function, no conclusions can be drawn about possible interactions at lower acetylsalicylic acid plasma concentrations approaching the sigmoid part of the concentration-effect-curve. However, it can be concluded that the antiplatelet effects of acetylsalicylic acid are not influenced by circulating salicylate during treatment with pharmacological doses of the drug.

The effects of both drugs on platelet function were compared in the second part of the study. Acetylsalicylic acid dramatically decreased thromboxane formation in both collagen and thrombin-treated platelets, while aggregation was impaired only when collagen was used for stimulation. This is in accordance with previous results showing that the activation of human platelets caused by higher doses of thrombin is not dependent on the cyclooxygenase pathway but results from phospholipase C mediated formation of phosphatidic acid from phosphatidylinositole which is then followed by release of arachidonic acid and subsequent formation of thromboxane and 12-HETE (Lapetina & Siess, 1983; Smith, 1980). In contrast to the observed effects of acetylsalicylic acid, salicylate administration, similarly to the first study, neither affected thromboxane release nor platelet aggregation when collagen or thrombin were used to induce aggregation.

Salicylate by itself is a potent non-steroidal anti-inflammatory and analgesic drug, pharmacological properties which usually have been thought to be related to inhibition of cyclooxygenase activity (Flower, 1974). Because inhibition of prostanoid formation has not been reported convincingly after treatment with salicylate, however, it has been suggested that other mechanisms might be involved in its anti-inflammatory properties (Atkinson & Collier, 1980). In order to clarify further the controversial role (Vane, 1978) of the arachidonic acid cascade in the pharmacological effect of salicylate, we now compared the effect of sodium salicylate on prostanoid formation with that of acetylsalicylic acid in doses used in the treatment of rheumatic diseases. In this part of the study both drugs were administered in the same molar dose leading to identical plasma salicylate concentrations. Prostanoid formation was assessed as platelet thromboxane synthesis as well as urinary excretion rates of PGE_2, an index of intrarenal prostaglandin formation (Frölich *et al.*, 1975), of the major metabolite of PGE_1 and PGE_2 (PGE-M) (Hamberg & Samuelsson, 1971; Rosenkranz *et al.*, 1983), a parameter of total body PGE formation (Hamberg & Samuelsson, 1971), and of the major metabolite of prostacyclin, 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Rosenkranz *et al.*, 1980). While acetylsalicylic acid significantly decreased urinary excretion of all measured prostanoids by about 60%, salicylate administration did not affect any of these values. This indicates that sodium salicylate in contrast to the acetylated compound does not influence prostaglandin E and prostacyclin formation *in vivo*.

In conclusion, our study demonstrates that administration of salicylate in a pharmacologically effective dosage in man does not interfere with the anti-platelet effect of acetylsalicylic acid. Furthermore, the non-steroidal anti-inflammatory salicylic acid has no discernable effect on cyclooxygenase in striking contrast to other drugs of this class such as acetylsalicylic acid. This suggests that its therapeutic effects are not mediated by inhibition of prostaglandin synthesis.

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