

Immunological Effects of Aspirin Anhydride, a Contaminant of Commercial Acetylsalicylic Acid Preparations¹

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Abstract. Aspirin anhydride (ASAN) has been found to be a frequent contaminant of commercial acetylsalicylic acid (ASA) preparations. ASAN is a highly immunogenic substance susceptible to form protein conjugates *in vitro*, to induce the formation of anti-aspiryl antibodies and of contact hypersensitivity to ASAN in guinea pigs and rabbits. Commercial ASA samples varied greatly in their capacity to induce hypersensitivity to ASAN under standardized conditions in guinea pigs. As demonstrated with various types of aspiryl-protein conjugates, antibodies induced by ASAN and detected by passive cutaneous anaphylaxis are mostly of aspiryl specificity.

Antibodies of similar specificity have been detected in patients ingesting aspirin. A few patients presenting a reliable history of generalized urticaria and acute angioneurotic edema after ingestion of aspirin showed skin hypersensitivity to aspiryl-polylysine. The possible significance of contamination of acetylsalicylic acid preparations by aspirin anhydride in the development of untoward reactions to aspirin is discussed.

It has been estimated that about 0.2% of the population presents some form of intolerance to aspirin [1, 2]. Whereas penicillin remains the drug most frequently causing untoward reactions, aspirin appears to be the origin of about 10% of all drug reactions being recorded in American hospitals [3]. Among clinical symptoms attributed to aspirin hypersensitivity, anaphylactic shock and generalized urticaria [4, 5, 7], bronchial asthma, rhinitis and nasal polyposis [6-8], gastric ulcerations and gastro-intes-

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tinal bleeding [9] have been emphasized. Acetylsalicylic acid has also been incriminated as cause of the renal lesions observed following abuse of analgetic drugs [10]. Whereas some of these symptoms, especially anaphylactic reactions, lead to suspect an allergic pathogenesis based on an antigen-antibody reaction, other symptoms of aspirin intolerance may not obligatorily be due to an immunological mechanism [7, 11]. Clinical and experimental data on aspirin intolerance have recently been thoroughly reviewed [12].

Experimental investigations on the immunochemical mechanisms by which aspirin preparations may form immunogenic protein conjugates and induce the formation of antibodies have been astonishingly few [12]. However, interest in aspirin intolerance has markedly increased in recent years [13-16, 21]. Two main conjugation pathways have been visualized, leading to the formation of:

(1) a N-acetyl determinant (fig. 1) through direct acetylation of protein carriers by the acetylsalicylic acid molecule; (2) a N-salicyloyl determinant (fig. 1) produced by the reactions of amines with salicyloyl acetic anhydride (fig. 1, III), an internal rearrangement product of the acetylsalicylic acid molecule.

Convincing evidence has been presented within the last years by FARR and his collaborators that acetylsalicylic acid is susceptible to acetylate proteins *in vitro* [17] and *in vivo* [18, 19]. Heavily acetylated proteins are immunogenic in experimental animals [20]. However, acetylation of albumin under 'physiological' conditions (ingestion) *in vivo* appears to involve at most 1,2 to 1,4 acetyl groups per molecule [18, 19] and there remains some doubt whether sizable amounts of antibodies against acetylated proteins may be found in patients ingesting aspirin. The evidence presented in this respect by MINDEN and FARR [22] has not yet been published in detail and has not been confirmed. Antibodies against N-acetylated albumin are obviously present only in very small amounts as they were not detectable by most classical immunological techniques but only by radioimmunoelectrophoresis.

On the other hand, the presence of antibodies specific for the N-salicyloyl determinant has been reported in man in several instances, generally using passive hemagglutination with proteins conjugated with aspiryl chloride or aspiryl azide [12, 23, 24]. However, the immunochemical pathway by which N-salicyloyl proteins may have been formed *in vivo* upon ingestion of aspirin has not been thoroughly investigated. Obviously, highly reactive aspiryl chloride frequently used to prepare aspiryl conju-

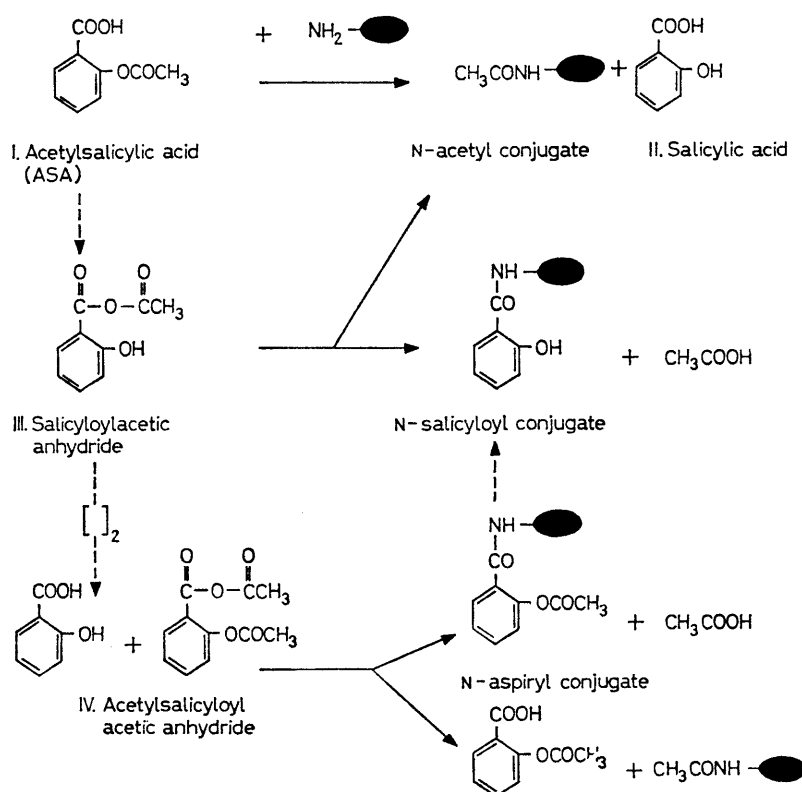


Fig. 1

gates *in vitro* is irrelevant to the problem of clinical aspirin hypersensitivity. Up to now it had been assumed on the basis of the suggestion of SCHWARZ and AMIDON [13] that the N-salicyloyl determinant arises directly from acetylsalicylic acid through an internal anhydride rearrangement (fig. 1, III).

It came to our attention [25] that procedures used in commercial synthesis of acetylsalicylic acid frequently promote the formation of aspirin anhydride (fig. 2, V) which then appears as an impurity in commercial preparations. Aspirin anhydride appeared to us to be much more likely than acetylsalicylic acid itself to react with proteins *in vivo* and to be responsible for the appearance of anti-salicyloyl and anti-aspiryl antibodies in patients ingesting aspirin. Acid anhydrides such as phthalic anhydride or

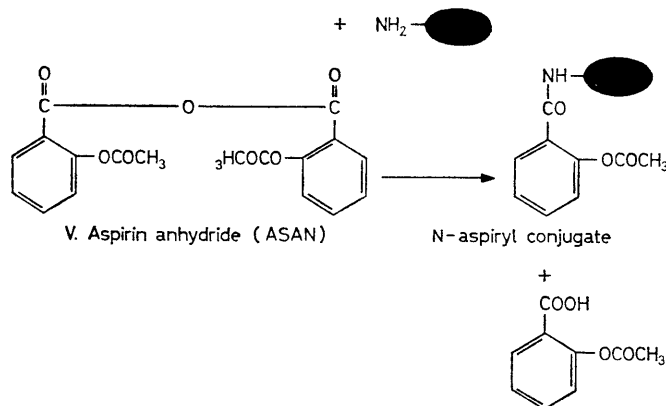


Fig. 2

citraconic anhydride are highly reactive with amines and are known to be potent immunogens [26]. Aspirin anhydride has been the object of numerous pharmaceutical and pharmacological studies [27–29] in the early sixties and has even been suggested as a substitute for aspirin. At that time, no attention seems to have been paid to its potential immunogenicity. Accordingly, we engaged in a study of the immunogenicity of aspirin anhydride and of its role in aspirin intolerance.

In this paper, we report the chemical demonstration of aspirin anhydride impurities in commercial acetylsalicylic acid samples, the conjugation of aspirin anhydride with proteins and polylysine *in vitro*, the immunogenic properties of such conjugates, of aspirin anhydride itself and of contaminated acetylsalicylic acid samples in experimental animals as well as some preliminary evidence on the potentially harmful role of aspirin anhydride impurities in man.

Material and Methods

Acetylsalicylic Acid and Other Reagents

Acetylsalicylic acid (ASA) in various degrees of purity and taken at various stages of the synthesis and purification procedure were made available to us by Chemische Fabrik Aubing, Munich, Germany. Acetylsalicylic acid samples from the current production of several manufacturers in various countries were purchased through commercial channels or made available by the manufacturers. Aspiryl chloride (ASPCL) was prepared by the method of WEIZMANN *et al.* [30], as modified by

WICHER *et al.* [14]. Acetic anhydride and succinic anhydride were purchased from Merck, Darmstadt, Germany. Aspirin anhydride (ASAN) and salicylic acid (SA) were from Chemische Fabrik Aubing. All organic solvents used were of reagent grade.

Polylysine hydrochloride (PLL) was prepared according to KATCHALSKI [31], as previously described [32]; average molecular weight was 3,200 according to internal viscosity measurement (n:21 lysine residues). Human serum albumin (HSA), bovine γ -globulin (BGG) and ovalbumin (OA) were from Pentex, Kankakee, Ill.

Analysis of Aspirin Anhydride in Acetylsalicylic Acid Samples

In order to detect the minute amounts of ASAN frequently present in commercial ASA samples and which always represent less than 1%, it is first required to concentrate the ASAN impurity by some kind of differential extraction procedure. In the method originally used by Chemische Fabrik Aubing [33], the ASA sample was dissolved in hot petroleum and recrystallized by reflux cooling whereupon ASAN remained in solution. However, this procedure promotes the formation of ASAN and of various other unidentified products, leading to erroneously high analytical contents of ASAN. On the other hand, small amounts of ASAN may be quantitatively extracted even in presence of a large ASA excess by dissolving the sample under vigorous stirring at room temperature in a mixture of equal volumes of benzol and 0.15 M phosphate buffer pH 7.8, 0.5 N sodium hydroxide being continuously added dropwise over a period of about 90 min in order to maintain the pH. Under these conditions, virtually no ASA passes into the benzol phase and the recovery of added ASAN is nearly complete (see results). After complete dissolution of the ASA sample, the benzol phase is concentrated *in vacuo* at room temperature after careful washing with water. Alternatively, similar results may be obtained by dissolving the ASA sample in ethyl acetate and 20% potassium bicarbonate at room temperature [33].

The benzol or ethyl acetate extracts were concentrated *in vacuo*, respectively dried and redissolved in acetone. The test solutions were applied on Kieselgel G thin layer chromatography plates (Merck, Darmstadt, Germany) and developed in various solvents (ethyl acetate-chloroform-formic acid 3:2:1; carbon tetrachloride-acetic acid 4:1 [33] or methylethylketone-cyclohexane 7:3 [33]. After development the plates were examined under ultraviolet light at 254 nm.

For colorimetric analysis of ASAN, the benzol extracts from ASA samples were reacted with a hydroxylamine reagent, heated in a water bath at 50 °C and the developed color measured in a Beckmann DB spectrophotometer between 520 and 530 nm. Further details on the analytical procedures will be published elsewhere.

Preparation of Aspiryl-Polylysine (ASP-PLL) with Aspirin Anhydride

321 mg of polylysine hydrochloride were dissolved in 7.5 ml distilled water and mixed with 7.5 ml freshly distilled tetrahydrofuran and 1.95 mg 1 N sodium hydroxide. Under vigorous agitation in a cooled water bath, 205.5 mg ASAN dissolved in 3 ml tetrahydrofuran were added in 3 portions over a 1-hour period, together each time with 1 ml 0.2 N sodium hydroxide in order to maintain the pH around 11. Following incubation with ASA, 300 mg succinic anhydride dissolved in 6 ml tetrahydrofuran were added in 3 portions over a 1-hour period, together with 2 ml 1 N so-

dium hydroxide each time. At the end of incubation, the pH was lowered to 8 with HCl and tetrahydrofuran distilled *in vacuo* at 25 °C. The remaining aqueous solution was diluted with the same volume of 0.05 M phosphate buffer pH 7.4 and centrifuged. The supernatant was chromatographed on a 300 ml (2.5 × 100 cm) Sephadex G-25 column, elution being performed with 0.01 M phosphate saline pH 7.4. Effluent fractions were collected and monitored spectrophotometrically at 305 nm.

Preparation of Aspiryl-Protein Conjugates with Aspirin Anhydride

Two-hundred milligrams ASAN were added to 500 mg of protein dissolved in 20 ml 0.1 M phosphate saline pH 7.4 and vigorously shaken for 24 h at 37 °C in a tightly stoppered flask. After incubation, the turbid solution was cleared by centrifugation and either dialyzed against distilled water followed by saline for 4 days or chromatographed on a Sephadex G-25 column.

Preparation of Aspiryl-Protein Conjugates with Aspiryl Chloride

Hundred milligrams protein were dissolved in 10 ml water and adjusted to pH 8.5–9 in a cold water bath. 100 mg aspiryl chloride dissolved in 1 ml ether were added slowly under stirring, the pH being maintained above 8.5 with 1 N NaOH. After periods of incubation varying between 30 min and several hours, the reaction mixture were dialyzed against distilled water for 48 h, followed by saline for additional 48 h.

Analysis of Aspiryl-Polylysine and Aspiryl-Protein Conjugates

Pooled fractions from column chromatography of ASP-PLL were analyzed for nitrogen by the colorimetric procedure of STEGEMANN [34] and for aspiryl groups by the molecular extinction at 305 nm ($E_M^{305}; 3,300$). For aspiryl proteins, the protein concentration was determined by the biuret reaction and the concentration of aspiryl groups by the increase in optical density at 305 nm.

Immunization with Aspiryl-Protein Conjugates

Albino guinea pigs from a closed colony weighing 300–400 g were immunized by injection of 0.1 ml in each footpad of an emulsion consisting in equal volumes of aqueous phase containing the aspiryl-protein conjugate in 0.01 M phosphate saline pH 7.4 and of complete Freund's adjuvant (Difco). Booster injections were administered 2 and 3 weeks later subcutaneously (0.1 ml) without adjuvant. The total amount of protein conjugate injected varied between 1 and 2 mg. A similar immunization schedule was used for rabbits of about 3 kg body wt., injecting 0.4 ml emulsion per footpad. The total amount of protein antigen injected in rabbits were between 5 and 7 mg. Bleeding by heart puncture was performed on the 28th day and at various intervals thereafter. All sera were cleared by centrifugation and kept frozen at –20 °C.

Immunization with Aspirin Anhydride and Acetylsalicylic Acid Samples

Immunization of guinea pigs or rabbits ASAN was achieved usually by repeated intradermal injections in the back of 0.1 ml ASAN dissolved in an organic solvent such as acetone, dimethylformamide or dimethylsulfoxide. Owing to the skin necro-

sis following such injections, the number of injections was usually limited to 5 on alternate days.

However, sensitization was also achieved by ASAN merely suspended in aqueous media and injected i.d. or by repeated epicutaneous applications in an organic solvent (table II). Details concerning the dose injected, the number and rhythm of injections and the date of bleedings are given with the corresponding experimental results.

Immunization of guinea pigs with various ASA samples was achieved following similar procedures, ASA being dissolved either in water or in dimethylsulfoxide (table III). No adjuvant was used in animals immunized with ASAN or ASA.

Detection of Anti-Aspiryl Antibodies

The presence of anti-aspiryl antibodies was investigated by immunodiffusion in agar, passive cutaneous and systemic anaphylaxis in guinea pigs and passive hemagglutination. Immunodiffusion was carried out in agar or agarose in small Petri dishes using a Shandon No. 1815 cutter. For passive cutaneous anaphylaxis, guinea pigs weighing 250–300 g were sensitized i.d. by 0.1 ml of serum dilution and challenged i.v. with 0.5 ml ASP-PLL (2 nmoles aspiryl/ml) and 0.5 ml 2% Evans blue 24 h later. In actively sensitized guinea pigs systemic anaphylactic shock was elicited by a similar dose of ASP-PLL and Evans blue.

For passive hemagglutination, aspiryl proteins (e.g. ASP-HSA) were bound to sheep or rabbit erythrocytes either with 2.5% glutaraldehyde [35] or with tannic acid according to BOYDEN [36]. Concentrations of proteins used varied between 5 and 30 mg/ml. The hemagglutination assay was carried out in microtitration plates according to TAKATSY [37].

Detection of Contact Hypersensitivity to Aspirin Anhydride in Guinea Pigs

For optimal results (fig. 8), 0.25 ml of a 1% and 5% (w/v) ASAN solution in acetone-almond oil (1:1 v/v) were applied with a pipette on stamped round 2 cm² surfaces of the flank, which had been shaved with an electric clipper some hours previously. Reactions were read after 24 h and evaluated according to the following scale:

- 0: No reaction
- 0.5: Disseminated faint red papules over the test area
- 1.0: Confluent erythema
- 2.0: Marked erythema and slight edema
- 3.0: Deep erythema, edema and necrosis.

In preliminary experiments (fig. 8) other solvents and concentrations were used. The maximal concentration of ASA to be dissolved in acetone-almond oil was 5%. Higher ASA concentrations could be achieved in pure dimethylsulfoxide, but this solvent was found to be slightly irritating and to make the reading difficult. The acetone-almond oil solvent generally used, on the other hand, had no primary toxic activity whatsoever.

The quantitative expression of the hypersensitivity level is given by the sum of all reaction grades observed in a group of animals and/or by the average of reaction intensity in animals tested with 1 and 5% ASAN concentrations.

Results

Detection of Aspirin Anhydride in Commercial Samples of Acetylsalicylic Acid

No analytical method specific for ASAN and directly applicable in presence of a large excess of ASA is available. Accordingly, traces of ASAN have first to be extracted from ASA samples and concentrated under conditions where ASAN may neither be produced from ASA (as apparently may occur in hot organic solvents) nor hydrolyzed to salicylic acid. Careful dissolution of ASA samples in a 2-phase system of phosphate buffer – benzol or potassium bicarbonate – ethyl acetate at pH 7.8–8.0 appears to fulfill these conditions. A highly pure ASA sample was mixed with ASAN in proportions varying between 0.2% and 4%. Under these conditions, the analytical procedure yielded practically quantitative recovery of ASAN. These results were confirmed by thin layer chromatography indicating that under the conditions used, no ASA from a very pure sample appears in the organic phase. The faint ASA spot observed in chromatography of ASAN samples (fig. 3) appears to be due to slight hydrolysis of ASAN in the organic phase. Whereas the quantitative colorimetric analysis of ASAN impurities appears to be reliable and re-

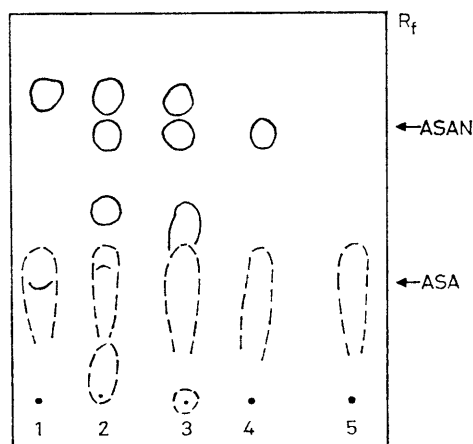


Fig. 3. Thin layer chromatography of ethyl acetate extracts of aspirin at pH 7.8. Development in methylethylketone-cyclohexane 7:3; (1) Commercial aspirin sample A; (2) Commercial aspirin sample B; (3) Commercial aspirin sample M; (4) Aspirin anhydride (ASAN); (5) Acetylsalicylic acid (ASA).

producibile at levels above 0.2%. The detection of ASAN impurities in ASA samples at levels between 0.01 and 0.1% has been attempted up to now only with thin layer chromatography. In that range, striking differences were encountered in the analysis of ASA samples originating from several manufacturers in various countries (fig. 3). ASA samples obtained from a manufacturer (Chemische Fabrik Aubing) using a synthesis procedure designed to minimize the presence of ASAN impurities in the final product appeared, in contrast to several other samples, as free of detectable ASAN.

Conjugation of ASAN with Proteins and Polylysine in vitro

As expected from an acid anhydride, ASAN appears to be highly reactive with amino groups. Under the conditions used (reaction of PLL with a sixfold molar excess of ASAN followed by succinylation of the remaining amino groups), about 50% of the available amino groups of PLL appear to be aspartylated. Under identical conditions but incubating PLL with a very pure ASA sample (Aubing) at about twice the molar concentration used for ASAN, no apparent conjugation occurred (fig. 4).

In order to establish whether ASAN would be susceptible to form biologically active conjugates with proteins under 'physiological' conditions, conjugates were prepared by shaking water insoluble ASAN at 37 °C with

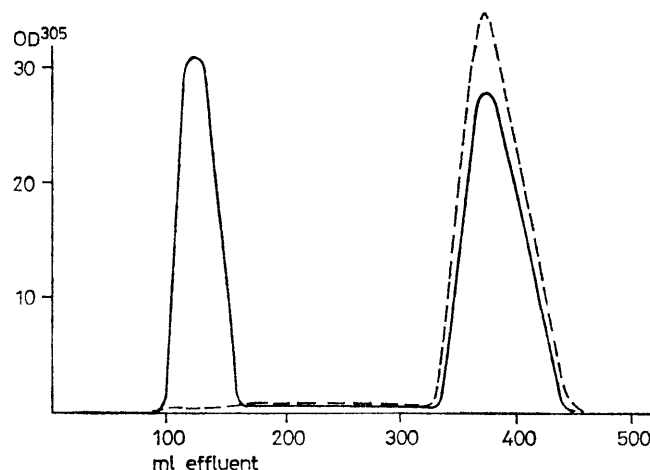


Fig. 4. Sephadex G-25 chromatography of: — ASAN incubated with PLL; ---- ASA incubated with PLL.

protein solutions. This is expected to reflect to some extent the type of contact which would occur in the digestive tract between ASAN and body proteins or food proteins upon aspirin intake. As shown in figure 5a, the formation of biologically active conjugates, namely of conjugates capable of eliciting specific PCA reactions, was clearly dependent upon the duration of incubation. In order to ascertain whether ASAN could also function as conjugating agent in presence of a large excess of ASA, ASA samples contaminated with various amounts of ASAN were used. Under the conditions used, the formation of PCA active conjugates was detected only in ASA samples containing 5% ASAN or more (fig. 5b). We could roughly estimate that the conjugating capacity of ASAN, its ability to form PCA active conjugates *in vitro* under these 'physiological' incubation conditions lies between that of the potent sensitizer dinitrochlorobenzene and of penicillin.

Immunological Properties of ASAN Protein Conjugates

As shown in table I, conjugates prepared by incubation of ASAN with various proteins induce the formation of antibodies specific not only for the protein carrier but also for an antigenic determinant shared by all ASAN-

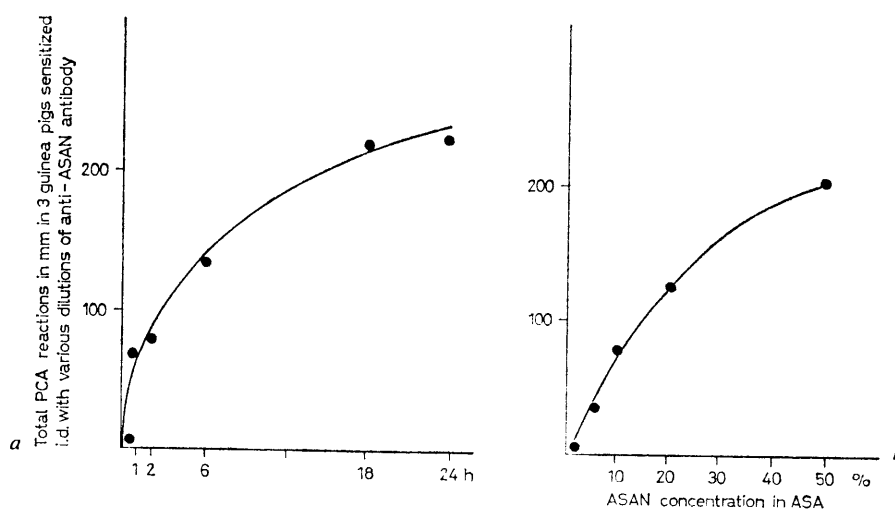


Fig. 5. *a* Effect of time of incubation upon the formation of PCA active ASAN-BGG conjugates, 37°C. *b* Effect of ASAN concentration upon the formation of PCA active ASAN-BGG conjugates, 37°C, 24 h.

Table I. Sensitization of guinea pigs to aspiryl-protein conjugates

Conjugate	Contact sensitivity to ASAN	Anti-ASP antibodies ¹	
		PCA	Shock
ASAN - BGG	0/11	11/11	6/6
ASAN - HSA	0/8	8/8	7/7
ASA (FRG I)-BGG	0/7	0/7	0/6
ASA (FRG I)-HSA	0/8	0/8	
ASA (Sweden)-HSA	0/5	0/5	
ASA (UK)-HSA	0/5	0/5	
ASA (FRG II)-HSA	0/5	0/5	
ASA (GDR)-HSA	0/5	0/5	

FRG: Federal Republic Germany

GDR: German Democratic Republic

UK: United Kingdom

¹ Detected with ASP-PLL.

Conjugates prepared by incubation at 37 °C, 24 h.

treated proteins and by aspiryl-polylysine. As shown in figure 6, an antiserum obtained from guinea pigs immunized with ASAN yielded PCA reactions with a whole series of aspiryl proteins and with aspiryl polylysine but not with proteins which had been incubated with pure ASA or with acetic anhydride. Conjugates prepared with aspiryl chloride were also effective in eliciting PCA reactions with antisera induced by ASAN or aspiryl proteins. Accordingly, there is little doubt that the antigenic determinant involved in these reactions is the aspiryl (fig. 2) or the salicyloyl (fig. 1) determinant.

Immunogenicity of Aspirin Anhydride in Guinea Pigs and Rabbits

ASAN dissolved in different solvents was administered to guinea pigs in various ways (intradermal, epicutaneous or per-oral). The development of hypersensitivity was followed by contact reactions with ASAN, by the detection of PCA antibodies and by the development of systemic anaphylactic hypersensitivity to ASP-PLL. The results of these experiments are shown in table II.

Following a standard immunization schedule, a dose-response curve was established (fig. 7). As may be seen, ASAN is a relatively potent im-

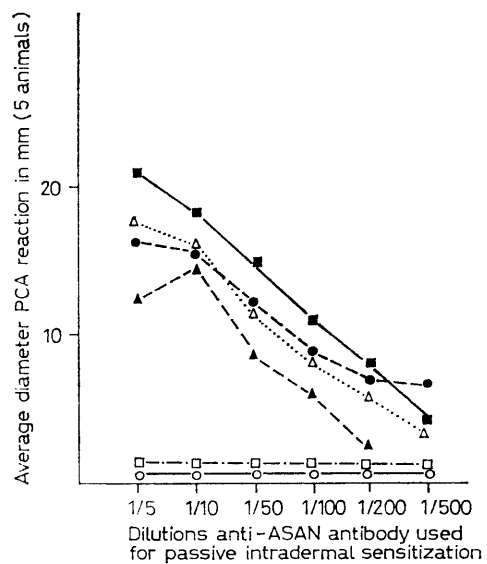


Fig. 6. Elicitation of PCA reactions by various types of aspiryl conjugates. ■—■ ASP-PLL (ASAN-PLL). ●—● ASPCI-HSA. △.....△ ASAN-HSA. ▲—▲ ASAN-BGG. □—□ ASA-HSA. ○—○ Acet-HSA.

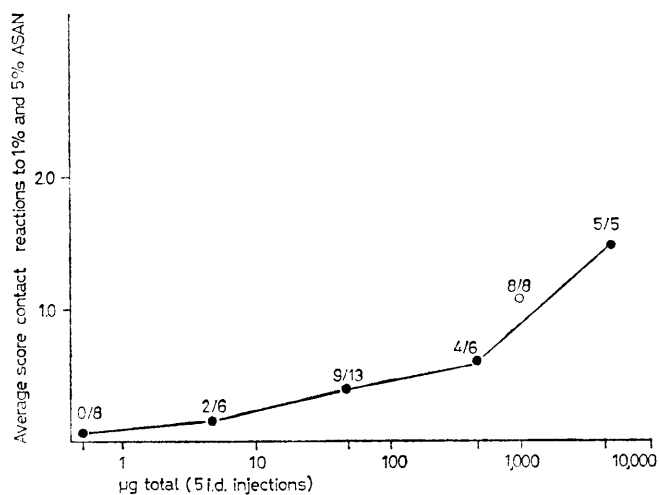


Fig. 7. Dose-response curve. Contact hypersensitivity to aspirin anhydride. ● ASAN in DMSO. ○ 1% ASAN in ASA (100 mg total).

Table II. Sensitization of guinea pigs to aspirin anhydride (ASAN)

Immunization mg	Diluent	Contact sensitivity to ASAN		Anti-ASP antibodies ¹	
		+ / tested	Score ²	PCA	Shock
5 × 20 i.d.	DMFO	8/8	2.1	7/8	
5 × 10 i.d.	DMFO	14/16	0.95		
5 × 1 i.d.	acetone	4/5	1.45	8/8	
5 × 0.1 i.d.	acetone	7/7	1.18	7/7	
5 × 0.01	acetone	5/7	0.39	2/6	
5 × 1 i.d.	DMSO	4/5	1.50	6/8	
5 × 0.1	DMSO	4/6	0.56	8/8	
5 × 0.01 i.d.	DMSO	9/13	0.38	7/15	5/8
5 × 0.001 i.d.	DMSO	2/6	0.12	0/8	1/6
5 × 0.0001 i.d.	DMSO	0/8	0	0/8	0/6
5 × 10 i.d.	H ₂ O	6/7	1.1	8/8	6/6
3 × 20 epi.	DMFO	8/8	1.5	1/8	
3 × 10 epi.	DMSO	12/13	1.0		1/10
5 × 1 i.d.	DMFO	8/8	2.4	6/8	4/4
5 × 25 epi.					
6 × 0.01 i.d.	DMSO	8/8	0.87	4/8	
1 × 15 epi.					
6 × 0.001 i.d.	DMSO	5/8	0.30	0/8	
1 × 15 epi.					
6 × 0.0001 i.d.	DMSO	2/8	0.09	0/8	
1 × 15 epi.					
10 × 200 <i>per os</i>	H ₂ O	2/5	0.20	0/5	

¹ Detected by ASP-PLL.² Score: average of all reactions of the animal group to epicutaneous tests with 1% and 5% ASAN in acetone-oil.

munogen which regularly sensitizes guinea pigs at doses over 500 μ g. Sensitization may be achieved either by intradermal injection or by epicutaneous application. The i.d. injection in organic solvents regularly causes a local necrosis but this necrosis and the resulting inflammation is not required for sensitization; similar results may be achieved with ASAN merely suspended in water. In this latter case, the development of delayed hypersensitivity may be followed by measuring the local infiltration at the injection sites, which regularly increases from the 7th day on. A few guinea pigs even appeared to develop contact hypersensitivity when adminis-

tered ASAN per os although in that case the reactions were very weak and somewhat doubtful. It should be remembered that feeding of a sensitizing hapten in guinea pigs usually results in immunological tolerance [38, 39].

Rabbits injected i.d. with ASAN developed antibodies detectable by PCA with aspiryl-polylysine. Rabbit anti-aspiryl antibodies were also detected in passive hemagglutination studies with aspiryl proteins prepared either with ASAN or with aspiryl chloride, but they did not yield positive results in agar double immunodiffusion. Antibody levels were apparently too low to permit precipitation.

Immunogenicity of Various Commercial Aspirin Samples

Various commercial ASA samples as well as ASA samples taken at various stages of the manufacturing process (condensation and recrystallization) were injected i.d. to guinea pigs in various solvents. The development of hypersensitivity was followed as described above from contact reactions to ASAN, from the presence of PCA antibodies and the development of systemic anaphylactic hypersensitivity to ASP-PLL. The results presented in table III show that various ASA samples are indeed capable of inducing contact skin hypersensitivity to ASAN and antibodies of aspiryl specificity. However, in these preliminary experiments, sensitization appeared erratic and some of the negative results obtained may have been due to the fact that the doses injected were too low.

Commercial ASA samples were then compared for their immunogenic capacity using higher doses and a standard immunization schedule (table IV). From these experiments, it became obvious that commercial ASA samples vary greatly in their capacity to sensitize guinea pigs to ASAN. The number of animals immunized with each sample is too small to establish a strict quantitative correlation between the traces of ASAN detected by thin layer chromatography and the capacity to sensitize guinea pigs under the conditions used. However, some rough correlation was apparent. It was obvious for example that the chromatographically 'ASAN-free' Aubing sample taken at the last manufacturing step after recrystallization markedly differed from other ASA samples and was not immunogenic under the conditions used.

As observed with the dose-response curve of ASAN as well as in other similar systems of hapten sensitization [40], contact hypersensitivity appears to be the most sensitive parameter indicating sensitization. The production of systemic anaphylaxis and of circulating antibodies detectable

Table III. Sensitization of guinea pigs to aspirin

ASA sample	Immunization mg i.d.	Diluent	Contact sensitivity to ASAN		Anti-ASP antibody ¹	
			+ / tested	Score	PCA	Shock
FRG A						
(condensation)	8 × 10	H ₂ O	0/4	0	0/5	0/5
FRG A	3 × 50	DMFO	4/7	0.39	6/8	8/8
(cond. + 1 % ASAN)	5 × 10	DMFO	6/7	0.39	6/8	8/8
FRG A	5 × 10	DMFO			0/8	
(recrist.)	² + 3 × 10	DMFO	2/7	0.14	0/7	0/7
FRG A	5 × 10	acetone			0/8	
(recrist.)	² + 3 × 10	acetone	8/8	0.56	5/8	6/6
FRG A	5 × 10	H ₂ O			0/8	
(recrist.)	² + 3 × 10	H ₂ O	0/8	0	1/6	2/6
Sweden	8 × 10	H ₂ O	0/4	0	0/4	0/4
UK	8 × 10	H ₂ O	0/4	0	0/5	0/5
FRG B	8 × 10	H ₂ O	3/3	1.0	0/5	0/3
GDR	8 × 10	H ₂ O	0/4	0	0/5	0/4

¹ Detected by ASP-PLL.² Epicutaneous test with 15 mg ASAN (non sensitizing in controls) between both injection series.

by PCA requires larger doses of sensitizing hapten. Accordingly, contact hypersensitivity to ASAN seems to be the most sensitive indicator of sensitization to aspirin.

Elicitation of Contact Reactions by Aspirin Anhydride and Commercial Aspirin Samples

The immunological relationship between ASAN and ASA could be evaluated not only from their relative immunogenicity, i.e. the capacity to induce antibody formation or contact hypersensitivity, but also from their respective ability to elicit contact reactions in already sensitive animals. As may be seen from figure 8, guinea pigs repeatedly injected with ASAN at doses varying between 1 and 5 mg show definite contact reactions to ASAN only at concentrations of 1% and more. Among the various solvents tested a mixture of equal volumes of acetone and vegetable oil appeared to give optimal results. Dimethylsulfoxide being slightly primary toxic was abandoned although it had the advantage of permitting the dis-

Table IV. Sensitization of guinea pigs to aspirin under standardized conditions (total dose 100 mg)

ASA sample	Contact sensitivity to ASAN		Anti-ASP antibodies ¹	
	+ / tested	Score	PCA	Shock
FRG A (condensation mixed with 1% ASAN)	8/8	1.07	8/8	8/8
FRG A (condensation)	7/25	0.09	0/8	2/25
FRG A (crystallized)	0/16	0	0/16	0/14
FRG B	5/8	0.47	0/8	0/8
GDR	7/8	0.78	0/8	0/8
Poland	6/8	0.69	0/8	0/8
UK	2/8	0.11	0/8	0/8
USA	4/7	0.28	0/7	0/7
France	2/8	0.09	0/8	2/8
Rumania	5/6	0.58	0/6	1/6
Egypt	3/5	0.45	0/5	1/5
Sweden	1/7	0.09	0/7	0/7

¹ Detected by ASP-PLL.

Score: average of all reactions of the animal group to epicutaneous test with 1% and 5% ASAN in acetone-oil.

Immunization schedule: 5 i.d. injections of ASA 20 mg in DMSO, on days 0, 2, 5, 8 and 12. Bleeding on day 15, epicutaneous test on day 16.

solution of higher concentrations of ASA. Using moderately hypersensitive guinea pigs, contact reactions elicited by a 5% (w/v) solution of pure ASA artificially contaminated with 1% ASAN were very slight and comparable in intensity to the reactions elicited by a 100-fold lower concentration of ASAN (0.05%). As in order to test directly the capacity of ASA to elicit contact reactions one is limited by ASA solubility, exquisitely sensitive guinea pigs will have to be used in order to compare effectively in this way commercial ASA samples contaminated with 0.1–0.01% ASAN.

The contact hypersensitivity to ASAN appears also to be of aspiryl or salicyloyl specificity. In guinea pigs sensitive to ASAN, contact reactions of similar intensity were elicited by 1 and 5% aspiryl chloride. On the other hand, no contact reactions to acetic anhydride have been observed in these animals.

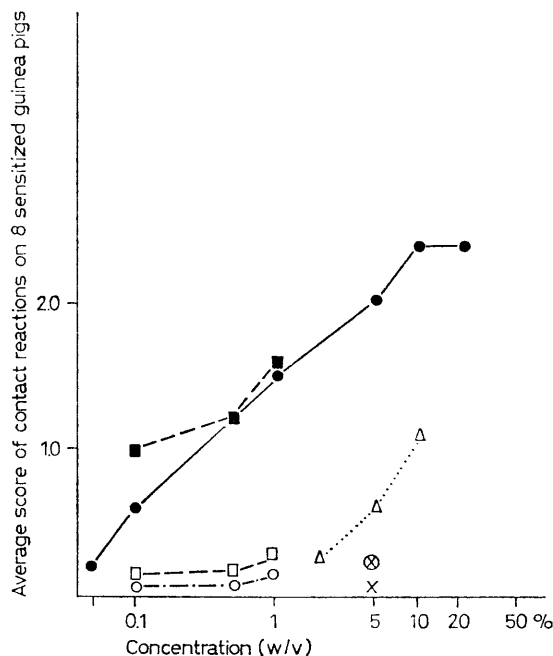


Fig. 8. Elicitation of contact reactions by various concentrations in guinea pigs sensitized to aspirin anhydride. ASAN in: □- - -□ acetone, ○- - -○ ethanol, Δ.....Δ DMFO, ■- - -■ DMSO, ●—● acetone-oil 1:1, ○—○ 1% ASAN in ASA, × ASA.

Detection of Hypersensitivity to the Aspiryl Determinant in Man

Although according to several reports, especially from the USA [3, 7] the incidence of intolerance to aspirin appears rather high, our limited experience indicates that aspirin hypersensitivity is relatively rare in Switzerland. Among 2,000 patients investigated in our Allergy Clinic in 1968 and 1969, only 32 qualified as possibly intolerant to aspirin from their history alone. Among these 32 patients, only 12 had a rather convincing history which was in some cases confirmed by exposition tests. Some of these patients and normal controls tolerating aspirin without apparent difficulty were skin tested with ASP-PLL. The results of these tests are given in table V. The following cases deserve to be described in more detail.

Case 1. D.A., male, 26 years old

This patient experienced within 1 month 2 episodes of angioneurotic edema and acute generalized urticaria 45 min to 1 h after ingestion of tablets containing 0.5 g of acetylsalicylic acid. The tablets were of different composition and the only com-

Table V. Skin tests to ASP-PLL

	ASA	Aspiryl-polylysine (ASP-PLL) (i.d.)						
	(scratch)	1:1	1:2	1:5	1:10	1:100	1:1000	1:10 000
A. <i>D. A., male, 26 years</i>								
Two episodes of acute angioneurotic edema and generalized urticaria, each time after ingestion of tablet containing ASA	∅	+++	++	++	+	(+)	-	-
<i>D. E., female, 29 years</i>								
Two episodes of acute angioneurotic edema, and generalized urticaria, once after ASA tablet, once after suppository containing ASA	∅	n.d.	n.d.	+++	++	+	(+)	(+)
<i>W. A., male, 54 years</i>								
Six episodes of acute angioneurotic edema and generalized urticaria after ingestion of tablets containing ASA and/or Pyramidon (skin test with Pyramidon +++)	++	n.d.	n.d.	+++	++	+	(+)	-
B. <i>Five patients with urticarial reactions to analgesics, dubious correlation with ASA</i>								
	{ 1 ∅ 4 ∅	++	+	+	(+)	-	-	-
		-	-	-	-	-	-	-
C. <i>Ten normal patients, without symptoms upon ASA intake</i>								
	{ 2 ∅ 8 ∅	(+)	(+)	-	-	-	-	-
		-	-	-	-	-	-	-

mon component was acetylsalicylic acid. Skin tests with ASP-PLL performed one month after the last acute allergic reaction were strongly positive.

Case 2. D.E., female, 29 years old

This healthy woman who had not experienced urticaria and angioneurotic edema before experienced within 2 months 2 episodes of acute generalized urticaria 1-2 h after intake of acetylsalicylic acid. The first time, the allergic reaction occurred after

ingestion of a mixed tablet containing acetylsalicylic acid, codein and phenacetin, the second time after administration of a suppository containing acetylsalicylic acid and pyramidon. The skin tests with ASP-PLL performed one month later were strongly positive.

Case 3. W.A., male, 54 years old

This man reported several episodes of acute angioneurotic edema and generalized urticaria following the ingestion of tablets containing pyramidon and/or acetylsalicylic acid. Skin tests with pyramidon and antipyrin-containing preparations were strongly positive. However, the administration of tablets containing only acetylsalicylic acid also led to generalized urticaria. Skin tests with ASP-PLL were strongly positive.

On the other hand, skin tests with ASP-PLL in 10 normal patients presenting no symptoms after aspirin intake were all negative although the undiluted preparation appeared to be slightly irritating in 2 patients. Hemagglutination studies were performed on the sera of our patients as well as on a number of sera made available by Prof. J. PEPYS (London), Prof. M. SAMTER (Chicago) and Dr. J. P. GIRARD (Geneva). Positive results were occasionally obtained but there is some question as to their specificity and no definite correlation with a reliable history of allergy to aspirin or with a positive skin test to PPL could be established for the time being in our patients. Further studies will have to be made on the specificity of antibodies detected by aspiryl-protein conjugates in human sera and on the relationship between hemagglutinating antibodies and clinical intolerance to aspirin.

A number of patients' sera were also investigated by passive cutaneous anaphylaxis in guinea pigs, using a latency time of both 4 and 24 h. Two sera among 40 examined yielded very strong and reproducible positive PCA reactions, which were elicited by ASP-PLL and other aspiryl-protein conjugates.

Discussion

Although aspirin is by far the most widely distributed and consumed drug since more than 70 years, its mode of action, the pathogenesis of several of its side effects and the nature of untoward reactions to aspirin are still largely unknown. Some of these reactions such as anaphylactic shock, angioneurotic edema or generalized urticaria certainly appear from a clinical point of view to be due to allergic hypersensitivity. Others, such as asthma, rhinitis and nasal polyposis associated with intolerance to aspi-

rin have for a long time also been ascribed to allergic hypersensitivity but other pathogeneses may also be visualized [7, 15]. Gastro-intestinal bleeding, which is one of the most common side effects of aspirin intake, has seldom been linked up to now to immunological phenomena but its pathogenesis is also unclear. Finally, no satisfactory mechanism has been established for the development of chronic interstitial nephritis among patients abusing of analgetic drugs. There too, an immunological mechanism could be postulated.

Among investigators concerned with the immunological properties of aspirin, two possible antigenic determinants have been considered. As demonstrated by FARR [17], acetylation *in vitro* may be effected directly by the aspirin molecule itself. Although acetylation of autologous proteins *in vivo* could lead theoretically to autoimmunisation and to the formation of antibodies against acetylated autologous proteins, the experimental evidence that antibodies against acetylated proteins frequently occurs in humans taking aspirin and that such antibodies are responsible for untoward clinical effects is not yet convincing. There is no doubt, however, that investigations along this line have to be pursued and that the observation now confirmed by several investigators [41-43] that aspirin is an effective acetylating agent *in vivo*, especially on platelets, has to be further evaluated.

On the other hand, the presence of antibodies of aspiryl or salicyloyl specificities in a certain proportion of patients taking aspirin appears to be a fact reported by several authors [12, 23, 24] although negative reports have been published [15]. As we show, some patients demonstrated skin hypersensitivity to aspiryl-polylysine and presumably possess skin sensitizing antibodies of aspiryl specificity. In these cases the presence of skin hypersensitivity to the aspiryl determinant appears to be linked with a clinical history of generalized reactions of anaphylactic type to aspirin, as would be expected. Whereas the specificity of anti-aspiryl antibodies detected in man by passive hemagglutination is possibly open to question (carrier specificity?), our finding of PCA positive sera with ASP-PLL seems to establish the fact that humans may possess anti-aspiryl antibodies.

Up to now it had been assumed that the formation of the aspiryl/salicyloyl determinant was due to some internal anhydride rearrangement of the aspirin molecule. This interpretation originates mostly from earlier chemical studies on the acid anhydride character of aspirin [44] and on the reactivity of aspirin with compounds possessing amino groups [45,

46]. However, our finding that aspirin anhydride as a reactive contaminant is present in most commercial acetylsalicylic acid preparations could well throw another light on these earlier reports. First, only a very small proportion of the acetylsalicylic acid used in incubation experiments with amino acids does actually form aspiryl derivatives. This amount was estimated by AMIDON and SCHWARZ [13] to represent 0.1–0.01%, which corresponds fairly well to the amount of aspirin anhydride possibly present in acetylsalicylic acid. Second, we have observed that the heating of acetylsalicylic acid in organic solvents definitely favours the formation of aspirin anhydride. Some of the reactions reported for aspirin [44] were performed under such conditions and may have been due to contamination with aspirin anhydride rather than to some internal rearrangement of acetylsalicylic acid itself.

The results presented in this paper demonstrate quite clearly that aspirin anhydride (ASAN) is a potent immunogen capable of reacting with molecules carrying amino groups, of sensitizing experimental animals in a variety of ways and of forming with proteins *in vitro* conjugates capable of inducing the formation of anti-aspiryl antibodies and of eliciting reactions of aspiryl specificity in sensitized animals. As already observed in other systems, contact hypersensitivity is the most sensitive biological assay to investigate the potential immunogenicity of a small allergenic chemical. From the dose-response curve established for contact hypersensitivity and from its capacity to substitute amino groups of proteins *in vitro*, ASAN seems to be somewhat less reactive than the classical experimental sensitizer dinitrochlorobenzene but is certainly a much stronger sensitizer than penicillin.

Although the specificity of antibodies induced in experimental animals by immunization with ASAN or in man by ingestion of aspirin has not yet been the object of extensive immunochemical investigations, there is little doubt that the antibodies detected by ASP-PLL or ASAN-protein conjugates are primarily specific for the aspiryl or salicyloyl determinant. This is indicated by the fact that ASP-PLL, ASAN-protein conjugates and conjugates prepared with aspiryl chloride readily cross-react in our system. The specificity for the aspiryl/salicyloyl determinant of antibodies induced by aspiryl chloride conjugates has been conclusively demonstrated [16]. Acetylation phenomena and specificity for the N-acetyl determinant were not detectable during immunization to ASAN. Guinea pigs contact sensitive to ASAN do not react to acetic anhydride and acetylated protein do not react with anti-ASAN antibodies. In the two human antisera which

were yielding strongly positive PCA reactions, cross-reactions were obtained with various types of conjugates prepared with ASAN or aspiryl chloride but not with acetylated proteins.

The finding that most commercial acetylsalicylic acid preparations contain ASAN in amounts varying between 0.01–0.1% suggests very strongly that the presence of anti-aspiryl antibodies in humans ingesting aspirin or in experimental animals being injected with aspirin is not due to an internal rearrangement of the ASA molecule itself but to the ASAN impurity. For this interpretation speaks on one hand the considerable difference in reactivity observed between ASAN and ASA as well *in vitro* (conjugation experiments) as *in vivo* (immunogenicity), on the other hand the fact that commercial ASA samples when injected into guinea pigs under standardized conditions vary greatly in their capacity to induce anti-ASAN hypersensitivity (table IV). Very pure ASA samples appear to be non immunogenic under these conditions. Difficulties in estimating very accurately the content of ASAN below 0.1% in ASA preparations does not permit for the time being to establish a strict quantitative correlation between the amount of ASAN impurity present in a given ASA preparation and its immunogenicity. However, and despite the relatively small number of animals used for immunization with each sample, a rough correlation with the results of thin layer chromatography was apparent. No adjuvant having been used and the animals being injected with identical doses under identical conditions and in parallel experiments performed during the same time period, it must be concluded that the variability of immunogenicity of ASA preparations is due to varying contents of ASAN and not to the ASA molecule itself.

Indeed, several questions remain to be answered. First, does the ingestion of traces of ASAN contained in ASA preparations suffice to sensitize humans? This question could only be answered with certitude by experiments in man using pure ASA preparations and preparations containing various levels of ASAN. However, the fact that several patients possess antibodies of aspiryl specificity and that in experimental animals antibodies of similar specificity are induced by ASAN and not by ASA suggests that even in man ASAN ingested together with ASA is responsible for anti-aspiryl hypersensitivity. Upon ingestion of 0.5 g ASA, 5–50 μ g ASAN are probably ingested, an amount far from being negligible and likely to be able to conjugate *in vivo* with autologous proteins or with food proteins and peptides. Second, it is not yet demonstrated that the majority of untoward reactions to aspirin are of immunological nature

and due to antibodies of aspiryl specificity. Indeed, it seems likely that some anaphylactic and other immediate-type reactions to aspirin are due to an immunological mechanism. As we have shown, some cases of repeated urticarial reactions after ingestion of aspirin possess a high degree of skin hypersensitivity of aspiryl specificity.

On the other hand, the clinical significance of hemagglutinating antibodies of aspiryl specificity is not yet clear. It may be recalled that a similar situation did exist at one time in penicillin allergy. The isolated appearance of hemagglutinating antibodies against penicillin was first thought to be devoid of any clinical significance because such antibodies were also found in non allergic patients [47]. It has been well established by now in the penicillin system that hemagglutinating antibodies reflect an immunogenic contact with penicillin even if no regular and absolute correlation with clinical reactions may exist. The reasons for the absence of parallelism between hemagglutinating antibodies and clinical allergic reactions to penicillin have been discussed in detail elsewhere [48]. It is possible that a similar situation applies to aspirin allergy. Another factor eventually responsible for the lack of correlation between presence of antibodies and clinical allergic reactions is the inbuilt inhibition [48] which will most certainly occur in the aspirin system. The excess of monovalent inhibiting aspirin in respect to the conjugates being formed by a trace impurity of aspirin anhydride may be more than sufficient to prevent in most cases clinical reactions. On the other hand, prolonged ingestion even in trace amounts of a highly reactive substance such as aspirin anhydride might not be without consequences especially on the renal parenchyma. This aspect certainly deserves further investigations.

Some of the untoward reactions to aspirin are most certainly not of immunological nature. The finding that acetylsalicylic acid prevents platelet aggregation probably by direct acetylation [41] apparently explains gastro-intestinal bleeding and the increased bleeding time observed in animals and man ingesting aspirin. In preliminary experiments using ASA preparations contaminated with various amounts of ASAN, we have attempted to evaluate whether the ASA molecule itself or the ASAN impurity is responsible for inhibition of platelet aggregation. It appears that the ASA molecule itself and not ASAN causes the inhibition effect on platelets.

Whereas pure ASA preparations will probably still cause untoward reactions, contamination of ASA preparations with ASAN represents a potential hazard to health and may be responsible for some of the untoward

reactions to aspirin. Accordingly, controls on the level of aspirin anhydride present in ASA preparations and fabrication procedures susceptible to minimize ASAN contamination should be fostered.

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Note added in proof: The presence of anti-aspiryl antibodies in humans has recently been confirmed by AMOS, H. E.; WILSON, D. W.; TAUSSIG, M. J. and CARTON, S. J.: Hypersensitivity reactions to acetylsalicylic acid. I. Detection of antibodies in human sera using acetylsalicylic acid attached to proteins through the carboxyl group. *Clin. exp. Immunol.* 8: 563 (1971).

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