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# Acetylsalicylsalicylic acid: a potentially immunogenic impurity in acetylsalicylic acid

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A study has been made of the chemical reactivity of a commonly occurring impurity in commercial acetylsalicylic acid preparations, acetylsalicylsalicylic acid. This impurity in aqueous solutions of pH 7·4–10·2 at 37° reacts with amines to produce salicylamide derivatives. The reaction rates are proportional to the concentration of amine in its free base form. Since the rates are appreciable even at physiological pH and the *N*-salicyloyl group has been reported to be an antigenic determinant in acetylsalicylic acid allergy, it is suggested that acetylsalicylsalicylic acid must be considered as a potentially immunogenic substance being involved in the development of allergic reactions to acetylsalicylic acid preparations.

Several authors have demonstrated the presence of antibodies of acetylsalicyloyl or salicyloyl specificities in man ingesting acetylsalicylic acid (Weiner, Rosenblatt & Howes, 1963; Giraldo, Blumenthal & Spink, 1969; Amos, Wilson & others, 1971; De Weck, 1971; Lazary, Toffler & De Weck, 1972), and in some persons a correlation between the presence of such antibodies and clinical allergic reactions to acetylsalicylic acid has been established (De Weck, 1971; Lazary & others, 1972). The development of acetylsalicyloyl/salicyloyl-specific antibodies in persons ingesting acetylsalicylic acid implies a formation of acetylsalicyloyl/salicyloyl-protein conjugates *in vivo* and up to now two conjugation pathways have been suggested: (i) a reaction between acetylsalicylic acid and free amino groups of protein, proceeding through a postulated anhydride rearrangement product of acetylsalicylic acid (Schwartz & Amidon, 1966), and (ii) a reaction between amino groups of protein and acetylsalicylic anhydride present as an impurity in most commercial acetylsalicylic acid preparations (De Weck, 1971). The acetylsalicylic anhydride was found to be a highly immunogenic substance capable of inducing the formation of acetylsalicyloyl/salicyloyl-specific antibodies in guinea-pigs and rabbits. Since commercial acetylsalicylic acid preparations varied greatly in their capacity to sensitize guinea-pigs under standardized conditions and pure acetylsalicylic acid samples, which had been freed of the anhydride by repeated recrystallizations, did not sensitize the animals under identical conditions, De Weck concluded that the immunogenic effect of acetylsalicylic acid is due to an acetylsalicylic anhydride impurity and not to acetylsalicylic acid itself.

Although the results obtained by De Weck (1971) show that a reactive impurity is responsible for the immunogenicity of the acetylsalicylic acid preparations and that the anhydride is a commonly occurring impurity possessing immunogenic properties, it has not been proved directly that the immunogenicity of the preparations is indeed due to the anhydride. Other impurities may be responsible for or at least may contribute to the immunogenic effect. Acetylsalicylsalicylic acid has recently been

found to be a frequently occurring impurity in commercial acetylsalicylic acid products (Patel, Perrin & Windheuser, 1972) and the experiments now described have been made to evaluate the chemical reactivity of this impurity with respect to ability to form *N*-acetylsalicyloyl or *N*-salicyloyl derivatives through reaction with protein-model amines.

## MATERIALS AND METHODS

### *Apparatus*

Ultraviolet spectra were recorded on a Perkin-Elmer 124 spectrophotometer and kinetic measurements were made on a Zeiss PMQ II spectrophotometer with a thermostatted cell compartment and a Servogor potentiometric recorder. pH was measured using a Radiometer Model PHM 26 pH meter.

### *Materials*

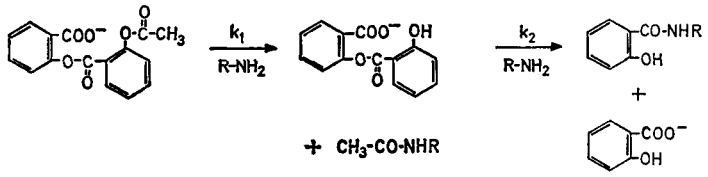
Salicylsalicylic acid was obtained from Fluka AG and was recrystallized from benzene. Acetylsalicylsalicylic acid was prepared by acetylating salicylsalicylic acid with acetic anhydride according to the general acetylation procedure of Chattaway (1931), m.p. 163–164° (from aqueous ethanol), lit. m.p. 161–162° (Einhorn, 1911). *N*-Salicyloylglycine and *N*-salicyloyl-ϵ-aminocaproic acid were prepared by aminolysis of phenyl salicylate as described by Schwartz & Amidon (1966). All other chemicals used were of reagent grade quality. Thin-layer chromatography was made with pre-coated silica gel $F_{254}$ aluminium sheets (E. Merck AG).

### *Kinetic measurements*

All kinetic measurements were carried out in aqueous solution at 37 ± 0·1°. Ionic strength was maintained at 1·0 by the addition of calculated amounts of potassium chloride. The reactions were performed in 3 ml aliquot portions of glycine buffer solution in a thermostatted spectrophotometer cell and were initiated by adding 10–20 μl of a stock solution of the esters in acetonitrile to give a final concentration of about $10^{-4}$M. The reaction progress was followed spectrophotometrically by recording the change in absorption at the wavelength which showed greatest difference in absorption of the ester and the reaction products. Aminolysis of acetylsalicylsalicylic acid (to yield salicylsalicylic acid) was followed at 340 nm and aminolysis of salicylsalicylic acid at 300 nm. For reactions at pH 7·4, however, the aminolysis was followed by recording the increase in absorption at 306 nm for acetylsalicylsalicylic acid and at 290 nm for salicylsalicylic acid. All the reactions observed displayed good first-order kinetic behaviour. The observed pseudo-first-order rate constants ($k_{obs}$) were calculated from plots of log ($A_\infty - A_t$) *versus* time, where $A_\infty$ and $A_t$ are the absorbance readings at infinite and at time *t*, respectively, or by the method of Guggenheim (1926). Each kinetic run was made in duplicate, the rate constants obtained therefrom being reproducible to within ±3%.

## RESULTS

The overall reaction of acetylsalicylsalicylic acid with the amines glycine and ϵ-aminocaproic acid was found to follow the pathway depicted in Scheme 1. The ultraviolet spectra of solutions in which the reactions were complete were identical with the spectra of artificially prepared mixtures of salicylic acid and the appropriate




*N*-salicyloyl amine. In addition identification of reaction products was accomplished using thin-layer chromatography (Table 1). The ultraviolet spectral changes accompanying the reaction of acetylsalicylsalicylic acid with glycine showed salicylsalicylic acid to be an intermediate in the process. During the reactions the absorbtion at 340 nm ($\lambda_{max}$ for salicylsalicylic acid at pH >9·6) was observed first to rise and then to decrease. The rate of formation of salicylsalicylic acid was always much greater (about 30 times) than the rate of its subsequent degradation and therefore when determining the kinetics of the initial process of the two consecutive reactions, this process could be treated as an ordinary one-step reaction. Due to aminolysis of the initially formed salicylsalicylic acid and the resulting inaccuracy of determination of the infinite absorbance points, the rate constants for the first reaction step were calculated by Guggenheim's method.

*Kinetics of aminolysis by glycine*

Under the conditions of glycine in large excess over ester, first-order kinetics were observed at constant pH. As can be seen from Fig. 1 the rate of the reactions of glycine with acetylsalicylsalicylic acid and with salicylsalicylic acid increases linearly with the concentration of glycine and also increases with pH. This shows that the reactions are first-order with respect to glycine concentration and that the glycine anion is the reacting form of the amino-acid. The reactions are kinetically described:

$$k_{1\ obs} = k_{1\ hydr} + k'_1\,(G_T) \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$k_{2\ obs} = k_{2\ hydr} + k'_2\,(G_T) \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where the numerals refer to the two reactions shown in Scheme 1; $k_{obs}$ is the observed pseudo-first-order rate constant, $k_{hydr}$ is the rate constant for hydrolysis (equal to the (small) intercepts of the lines in Fig. 1), k′ is the pH-dependent apparent second-order rate constant for the glycinolysis (equal to the slopes of the straight lines in Fig. 1) and ($G_T$) is the total glycine concentration. The true second-order

Table 1. $R_F$ *values for various salicyl compounds.*

| Compound | Solvent I | Solvent II |
|---|---|---|
| Acetylsalicylsalicylic acid | 0·60 | 0·27 |
| Salicylsalicylic acid | 0·65 | 0·36 |
| Salicylic acid | 0·49 | 0·30 |
| *N*-Salicyloylglycine | 0·31 | 0·05 |
| *N*-Salicyloyl-ε-aminocaproic acid | 0·56 | 0·18 |

Solvents: I = glacial acetic acid—ethyl acetate (3:97); II = glacial acetic acid—benzene (1:9). Silica gel $F_{254}$ sheets were used and the compounds observed under 254 nm ultraviolet radiation.

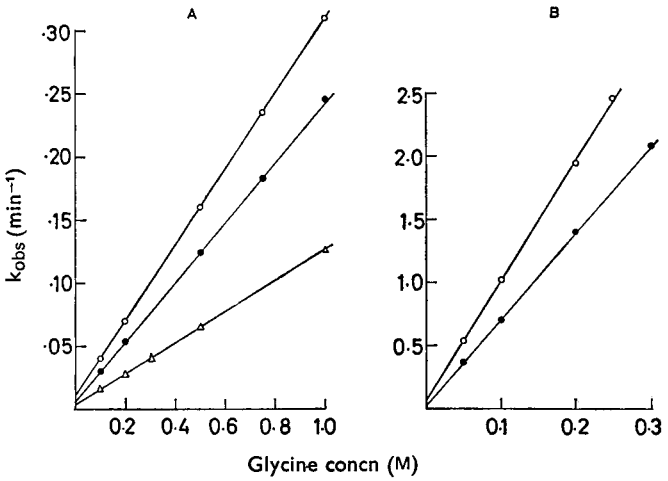



FIG. 1. Plots showing the dependency of the observed pseudo-first-order rate constants for the aminolysis of salicylsalicylic acid (A) and acetylsalicylsalicylic acid (B) upon total glycine concentration at 37° and at pH 9·14 (△), pH 9·66 (●), and pH 10·15 (○).

rate constants $k_1$ and $k_2$ for attack by glycine in its free base form were calculated from equations 3 and 4:

$$k_1 = k_1' \frac{K_a}{K_a + a_H} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

$$k_2 = k_2' \frac{K_a}{K_a + a_H} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

where $a_H$ is the hydrogen ion activity as determined by the glass electrode and $K_a$ is the ionization constant of the conjugate acid of the glycine amino group (equal to $10^{-9.49}$ at 37° and $\mu = 1{\cdot}0$ by potentiometric titration). The values found were:

$$k_1 = 0{\cdot}39 \pm 0{\cdot}04 \text{M}^{-1}\,\text{min}^{-1}\ (37°;\ \mu = 1{\cdot}0)$$

$$k_2 = 11{\cdot}4 \pm 0{\cdot}3 \text{M}^{-1}\,\text{min}^{-1}\ (37°;\ \mu = 1{\cdot}0)$$

At pH 7·4 and 37°, and with a total glycine concentration of 1·0M, the calculated half-lives for reactions 1 and 2 are 7·5 min and 220 min, respectively, which agree with experimentally determined values of 8 min and 210 min, respectively. The last value has been corrected for the simultaneously proceeding hydrolysis of salicylsalicylic acid, the rate of which represented 10% of the overall reaction rate.

## DISCUSSION

The recent observation by De Weck (1971) that the sensitizing capacity of commercial acetylsalicylic acid preparations varies widely, strongly suggests that the sensitizing effect is due to an impurity and not to the acetylsalicylic acid itself. De Weck has detected acetylsalicylic anhydride in most of the preparations in amounts considered to lie within the range of 0·01–0·1%, and since the anhydride was found to be a potent immunogen capable of inducing the formation of acetylsalicyloyl- or salicyloyl-specific antibodies in animals, and antibodies of similar specificity were detected in patients ingesting acetylsalicylic acid, De Weck concluded that acetylsalicylic anhydride is the responsible immunogenic impurity. The occurrence of the anhydride as a frequent

contaminant of commercial acetylsalicylic acid preparations has also been reported by Bundgaard & Bundgaard (1973); the amounts found in their study of nine different preparations ranged from 0·0012 to 0·024%.

The present work indicates that another possibility for the *in vivo* formation of immunogenic *N*-salicyloyl-protein conjugates has to be taken into account. Patel, Perrin & Windheuser (1972) have recently detected acetylsalicylsalicylic acid in six different commercial acetylsalicylic acid preparations in amounts ranging from 0·02 to 0·13%, and the present examination of the chemical reactivity of this impurity shows that it is able to react with protein-model amino compounds with the formation of *N*-salicyloyl amines. The rate of the aminolysis by glycine depends on the concentration of the free base form of the amine but even at pH 7·4 the rate of reaction is appreciable. Obviously reaction rates measured *in vitro* may not be related to rates of reaction with proteins in the body but comparison with reactions known to be of immunological significance may be valuable. Penicilloylation of ε-amino groups of lysine residues of proteins through a direct reaction between penicillins and protein is assumed to be a route leading to formation of immunogenic penicilloyl-protein conjugates (see e.g. Schneider, 1970) and as an *in vitro* model of this reaction the reaction of penicillins with glycine has been studied kinetically by Schwartz & Wu (1966). At pH 7·4 and 50°, and with a glycine concentration of 1·0M, the half-life of aminolysis of benzylpenicillin is 13 h while the half-life of the reaction of glycine with acetylsalicylsalicylic acid to produce *N*-salicyloylglycine under the same conditions, except at a lower temperature (37°), is 3·5 h. Thus, it seems reasonable to conclude that acetylsalicylsalicylic acid must be considered as a potentially immunogenic substance capable of forming *N*-salicyloyl-protein conjugates *in vivo*. As the rate-determining step in the overall aminolysis of acetylsalicylsalicylic acid is aminolysis of the intermediate salicylsalicylic acid, this compound, too, may be immunogenic.

The formation of small amounts (0·01–0·1%) of salicylamides when acetylsalicylic acid was allowed to react with glycine and ε-aminocaproic acid in basic aqueous solutions as has been reported by Schwartz & Amidon (1966) may be due to a reaction between the amines and an acetylsalicylic anhydride impurity as pointed out by De Weck (1971), but as will appear from this study the formation can equally well be explained as arising from aminolysis of an acetylsalicylsalicylic acid impurity.

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