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CHEMICAL APPROACH TO ASPIRIN HYPERSENSITIVITY

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Summary: Rabbits and guinea pigs were immunized with functionalized aspirin-protein conjugates prepared by coupling 5-N-Succinylamino aspirin to BSA and BGG using a water soluble carbodiimide (EDC). Two populations of antibodies, one specific to functionalized aspirin and the other exclusively specific to salicylic acid were detected. These antibodies were fractionated and separated on affinity polymers suitably prepared with 5-N-succinylamino salicylic acid and 5-N-succinylamino-2-ethoxy benzoic acid as the ligands. The isolated and purified antibodies were electrophoretically homogeneous. The physico chemical interactions between the antibodies and the respective haptens were studied by radio-immunoassay, equilibrium dialysis and fluorescence quenching techniques.

Introduction: Inspite of intensive investigations(1-9) on aspirin hypersensitivity, opinions still differ regarding the immunological or non immunological nature of the syndrome. The difficulty in probing into the immunogenicity of aspirin has been associated with the highly labile nature of the molecule. Earlier attempts(7,10-12) to raise structure specific antibodies to aspirin by immunization with aspiryl protein conjugates prepared by coupling aspirin to the carrier protein through the aspiryl carboxyl group under alkaline conditions resulted in two serious problems: (a)The carrier protein is likely to mask the functional groups namely the O-acetyl and the carboxyl, which may be needed for the haptenic recognition by the lymphocyte cells, and (b)the hydrolysis of aspirin to salicylic acid during immunogenesis. The aspiryl carboxyl group coupled to the

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carrier protein, from a topological point of view, should elicit antibody reaction with any ortho disubstituted benzene hapten.

In the present investigation free exposure of the probable antigenic determinants of aspirin was accomplished by the functionalization of the aspirin molecule at position-5 of the aromatic ring through the introduction of an N-succinylamino group. The functionalized aspirin hapten was coupled to the carrier protein by using a water soluble carbodiimide(EDC). It was also used as ligand in the purification and the isolation of the structure specific antibodies by the application of affinity chromatography.

Materials and Methods: Preparation of 5-N-succinylamino aspirin-protein conjugate (Chart 1): A solution of 5-N-succinylamino aspirin(hapten I, 150 mg) in the minimum volume of cold sodium bicarbonate was acidified to pH 6.0 (1N HCl) and was stirred with the gradual addition of solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 100 mg) during 10 minutes. To the reaction mixture, BSA (100 mg) or BGG (80 mg) in 2 ml normal saline was added all at once and stirred for 2h at room temperature. The reaction product after dialyzing at 0° C against double distilled water was lyophilized and stored at -20° C.

Preparation of the affinity polymers: Three affinity polymers making use of Sepharose-4B, polyacrylamide and chloromethylated polystyrene matrices were functionalized by the standard methods (13) and linked to an ethylene diamine spacer arm. The ligand (5-N-succinylamino salicylic acid or (5-N-succinylamino-2-ethoxy benzoic acid) (300 mg) was added to the aminoethyl polymer suspension (10 ml packed volume) at pH 6.0 and treated with EDC (200 mg). The mixture was stirred at room temperature for 6h and for another 6 to 8h at 4° C. The gel was filtered and washed with two 50 ml aliquots of 0.05M sodium bicarbonate, 0.05M acetic acid and water.

Immunization: Six albino rabbits (3 male & 3 female) and six guineapigs (3 male & 3 female) were immunized, each with weekly injections of a suspension of 1 mg of the functionalized aspirin-protein conjugate in complete Freunds' adjuvant (0.5 ml) injected subcutaneously and intracutaneously at multiple sites on the back, thighs and groin. After 7 weeks the rabbits were bled by ear-vein puncture and the guineapigs were bled by heart puncture. The antisera were absorbed with the native, as well as the EDC treated carrier proteins to remove the carrier specific antibodies. The absorbed antisera were then chromatographed sequentially over the affinity polymer linked with 5-N-succinylamino-2-ethoxy benzoic acid and then over another affinity polymer linked with 5-N-succinylamino salicylic acid in Tris-HCl buffer

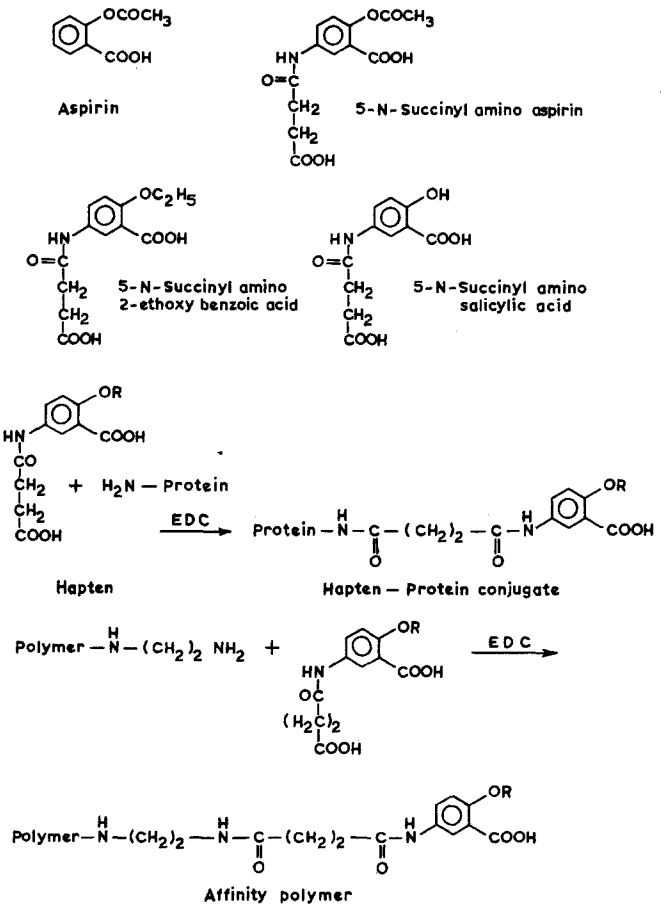


Chart 1

(0.05M, pH 7.2). After washing the polymers with saline buffer the antibodies were desorbed with 0.9M thiourea and 8M urea. Most of the antibody fraction was eluted out in 0.9M thiourea. Immunodiffusion tests were conducted with the antibodies eluted from the two affinity polymers according to the method of Ouchterlony (14), using the protein conjugates of 5-N-succinyl-amino aspirin (hapten I); 5-N-succinylamino salicylic acid (hapten II) and 5-N-succinylamino-2-ethoxy benzoic acid in borate saline buffer (pH 7.8).

Equilibrium dialysis and the hapten binding inhibition experiments were carried out using 5-N-(1,4 ^{14}C)succinylamino aspirin and 5-N-(1,4 ^{14}C)succinylamino salicylic acid as the labelled haptens and different structural analogues of functionalized aspirin (15). Fluorescence quenching studies were carried out according to the method of Eisen and Sirkind (16).

Result and discussions: The number of aspirin residues permole of the carrier protein was found to be $\sim$ 20 in the conjugate pre-

pared by the EDC coupling method and this method prevents the hydrolysis of aspirin to a large extent. However, the hydrolysis could not be totally prevented. As a consequence, the formation of heterogeneous populations of antibodies with specificities to the aspirin and salicylic acid moieties was envisaged. The use of functionalized aspirin as ligand on the affinity matrix was ruled out because of its ready lability. Hence a purely chemical strategy for trapping the aspirin specific antibodies was resorted to, by employing 5-N-succinylamino-2-ethoxy benzoic acid, a chemically stable analogue of the functionalized aspirin molecule as ligand on the affinity matrix(as a pseudo hapten). The antibody eluted from this affinity column(antibody I) gave precipitin bands with the protein conjugates of 5-N-succinylamino aspirin (hapten I); 5-N-succinylamino salicylic acid (hapten II) and 5-N-succinylamino-2-ethoxy benzoic acid. On the other hand the antibody eluted from the affinity polymer linked with 5-N-succinylamino salicylic acid(antibody II) gave precipitin band with the protein conjugate of hapten II and only a feeble band with the protein conjugate of hapten I, but none with the protein conjugate of 5-N-succinylamino-2-ethoxy benzoic acid. It can be thus inferred that the antibody I is more specific to the functionalized aspirin hapten but shows cross-reaction with the functionalized salicylic acid derived from hapten I, while the antibody II is specific exclusively to the functionalized salicylic acid molecule.

Fluorescence quenching experiments indicated that the antibody I(Q_{max}=52) is a low affinity antibody, with a heterogeneity index $\sim$1 where as the antibody II(Q_{max}=65) is of comparatively higher affinity with a heterogeneity index $\sim$ 0.6. Table I depicts the kinetic and the thermodynamic parameters such as the

Table 1

Comparison of the physico chemical parameters of the antibodies I and II+

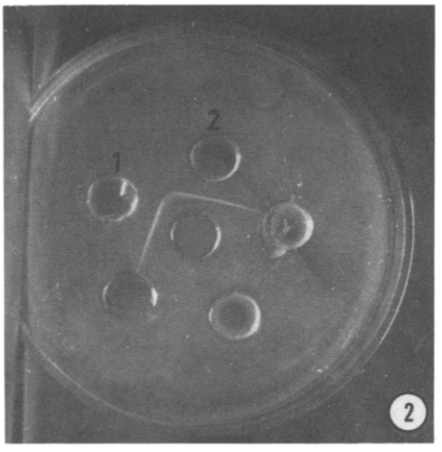
Physico chemical Parameter	Antibody I	Antibody II
Stoichiometry of binding with hapten I (5-N-Succinylaminoaspirin)	1 : 1.9	1 : 1.43
Stoichiometry of binding with hapten II (5-N-Succinylamino Salicylic acid)	1 : 1.8	1 : 1.88
++K.rel with hapten I	0.89	0.1
K rel with hapten II	0.35	0.85
K rel with aspirin	0.12	0.06
K rel with salicylic acid	0.10	0.18
Association constant (Ka) at 27° C	1.764×10^6 M^{-1}	7.596×10^6 M^{-1}
Association constant at 5° C	7.76×10^5 M^{-1}	2.68×10^6 M^{-1}
Change in free energy (ΔF) at 27° C (K.cals/mole)	-8.631	-9.507
Change in free energy (ΔF) at 5° C (K.cals/mole)	-6.2216	-8.231
Enthalpy (ΔH) K.cals/mole	-6.2216	-7.897
Change in entropy (ΔS) eu at 27° C	8.03	5.36
Q max	52.0	65.0

+ The data were derived from the hapten binding inhibition studies, Equilibrium dialysis and Fluorescence quenching experiments with the respective antibody (80mg) and known graded concentrations of the respective haptens.

K rel = Relative association constant and is given by the following expression.

$$\text{K rel} = \frac{\text{Molar concentration of the inhibitor required for 50\% hapten binding inhibition}}{\text{Molar concentration of the reference hapten required for 50\% hapten binding inhibition.}}$$

association constants(Ka), enthalpy(ΔH), change in free energy (ΔF), and the change in entropy(ΔS) of the hapten antibody interactions involving the antibodies I and II. From the Ouchterlony gel diffusion patterns (figures I and II) and the equilibrium



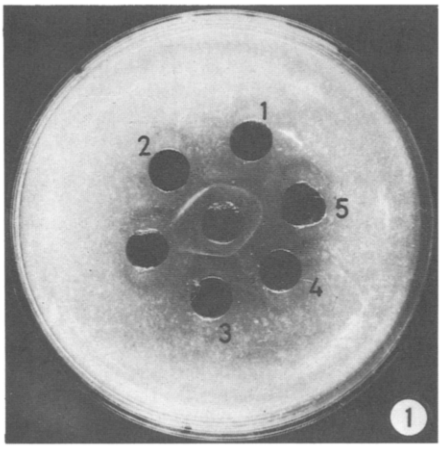


Fig.I : Ouchterlony patterns of antibody I
central well: antibody I

1,2 : 5-N-succinylaminoaspirin-protein conjugate

3,4 : 5-N-succinylamino-salicylicacid-protein conjugate

5 : 5-N-succinylamino 2-ethoxy benzoic acid-protein conjugate

Fig.II : Ouchterlony patterns of antibody II
central well: antibody II

1,2 : 5-N-succinylamino salicylicacid and conjugate

unnumbered wells : 5-N-succinylamino-2-ethoxy benzoic acid-protein conjugate

dialysis data it can be concluded that the same attachment pattern is present in the hapten binding sites of the antibody I for both the aspirin and salicylate haptens, one fitting somewhat better than the other. On the other hand the hapten binding site on the antibody II may have a polar recognition site for the aromatic hydroxyl group which the aspirin molecule lacks resulting in a greater specificity of the antibody II.

Functionalization of the hapten does not bring in a large difference in the specificity of the antibodies between the functionalized and the free haptenes as indicated by the relative

association constants(Table I). Attempts to locate the presence of a carrier receptor type of protein specific to aspirin [as was discovered in the case of Penicillin(17)] in the immunesera of the experimental animals were unsuccessful. The possibility of the existence of such a protein which might play an active role in the mediation of aspirin hypersensitivity is not ruled out although the experimental limits could not prove its existence. The elicitation of the separate aspirin specific and salicylic acid specific antibodies and the development of an affinity chromatographic technique to monitor the isolation of these antibodies in pure state might throw new light on the hitherto unsolved phenomenon of aspirin hypersensitivity. It is possible that in clinical aspirin immunogenesis, the molecule may undergo 5-hydroxylation which would lead to antigens analogous to hapten I.

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