Delayed Hypersensitivity to Hapten-Protein Conjugates. III. Saccharides as Haptens.* (28648)

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Heidelberger and coworkers(1) and Kabat (2) have established that polysaccharides can operate in certain instances as antigens capable of inducing antibody formation. In addition to the customary *in vitro* antigen-antibody reactions, the polysaccharide antigens mediate as well the typical *in vivo* hypersensitivity phenomena based upon the union of antigen with antibody, such as passive systemic anaphylaxis in the guinea pig(3) and the Arthus reaction in the rabbit(4). Goebel and Avery(5,6) also showed that monosaccharides such as glucose and galactose, when coupled to proteins as haptens, could serve as antigenic determinants in induction of antibody formation, while Ovary and Karush (7) showed that a saccharide hapten could also elicit immediate hypersensitivities.

In striking contrast to these results has been the consistent inability to elicit the delayed hypersensitivity reaction with polysaccharide antigens. Purified pneumococcal polysaccharide would not evoke a positive delayed skin or a corneal reaction in the immunized rabbit(8,9) unless the fraction contained protein or polypeptides. A similar study of the degradation of glycopeptide antigens from the fungus trichophytin showed that the saccharide moiety seems to function only in the immediate hypersensitivity responses, with a polypeptide component an obligatory participant in the delayed reaction(10).

With this background, it becomes pertinent to enquire whether the inability of a polysaccharide to mediate the delayed hypersensitivity response is due to its inability to serve as the haptenic focus of an antigenic determinant in the delayed system, or whether perhaps the fault lies elsewhere. This question assumes additional importance in view of the postulate by Karush and Eisen(11) that saccharide haptens should be ineffective in the delayed system by virtue of an inability to furnish enough binding energy to mediate the response.

The investigations described here have tested the capacity of saccharide haptens to function as determinants in the delayed hypersensitivity system, and the results indicate that they fulfill this function as effectively as do ionic and nonpolar haptens.

Materials and methods. Guinea pig albumin (GpA) was prepared by the method given in(12). Hen ovalbumin (Oval) was a recrystallized commercial preparation.

p-Aminophenyl-β-D-glucoside and *o*-aminophenyl-β-D-galactoside were prepared from the corresponding commercial nitrophenyl derivatives, purified, diazotized, and conjugated with either GpA or Oval by a procedure analogous to that of Goebel and Avery(5). *p*-Aminophenyl-β-lactoside (obtained through the courtesy of Prof. M. M. Rapport, Albert Einstein School of Medicine, New York) and *p*-aminophenylethyl alcohol (a purified commercial preparation) were coupled to proteins in a similar way. In every instance, the conjugation was made with a theoretical 60 haptenic groups per mole of protein. The coupling reaction was carried out in 0.2 *M* carbonate-bicarbonate buffer at pH 9.1, with stirring at 4°C overnight followed by dialysis against tap water. Samples of saccharide-protein conjugates were hydrolyzed by heating with an equal volume of *N* HCl for 3 hours in boiling water, treated with 10% sodium tungstate, and centrifuged to remove precipitated protein. Analyses of the supernates by the modified Folin-Wu method(13) showed the conjugates to possess an average of 3% reducing sugar, or 10 moles of hapten per mole of protein.

α-D-glucuronic acid and α-D-galacturonic acid were conjugated with GpA and with

* This work was supported by a contract from Medical Research and Development Command, U. S. Army.

TABLE I. Delayed Sensitivity Reactions to Monosaccharide-Azo-GpA Conjugates.

Sensitizing antigens	Test antigens			
	p-Azophenyl-glucoside-GpA	*o*-Azophenyl-galactoside-GpA	*p*-Azophenyl-ethyl alcohol-GpA	*p*-Azophenyl-glucoside-Oval
	Avg skin reaction diameter (mm)			
p-Azophenylglucoside-GpA				
Not desensitized	14	7	8	0
Desensitized with *o*-azophenyl-galactoside-GpA	13	0	7	0
Desensitized with *p*-azophenylethyl alcohol-GpA	13	6	0	0
o-Azophenylgalactoside-GpA				
Not desensitized	6	14	11	0*
Desensitized with *p*-azophenyl-glucoside-GpA	0	11	6	0*
Desensitized with *p*-azophenylethyl alcohol-GpA	6	10	0	0*

* It will be noted that in these controls both hapten and carrier were heterologous.

Oval by the mixed anhydride method(14), with isobutyl chlorocarbonate as the reagent forming the anhydride with the tri-*n*-butylamine salt of the uronic acid. Isobutyl carbamate-GpA conjugates were prepared for control experiments by the reaction of isobutyl chlorocarbonate in dioxane with a GpA solution at pH 9.1. Determination of free amino groups remaining in the uronic acid-protein conjugates by the ninhydrin method (15) showed that an average of 80% of lysine-ϵ-amino groups were substituted by isobutyl carbamate groups, apparently as a result of a side reaction during the conjugation procedure.

For the sensitization of guinea pigs, suitable dilutions of the protein conjugates in isotonic saline were emulsified with an equal volume of Freund's complete adjuvant (Difco). The immunizing dose, 10 μg, was contained in 0.2 ml of emulsion, half of which was injected into each rear footpad. Six animals were used for each sensitizing antigen. Albino guinea pigs of the Hartley strain, weighing 350 to 500 g, were used throughout.

On the 10th day after sensitization, the animals were tested intradermally in the flank with 10 μg doses of 4 different antigens, each contained in 0.1 ml of isotonic saline. Controls included the injection of sensitized animals with GpA alone and injections of unsensitized animals with saccharide-GpA conjugates.

The test sites were observed at 2 to 5 hours and then at 18 to 24 hours after challenge. The readings were recorded after 22 to 24 hours as the average diameters in millimeters of the erythematous lesions.

Following the reading of the tests, the animals were desensitized in groups of 3 by intraperitoneal injections of 5-mg doses of the appropriate antigen. Three to 4 hours later, the animals were retested intradermally in a fresh area of the skin, and the new reactions were read the next day.

Results. Groups of 6 guinea pigs were sensitized with either *p*-azophenylglucoside-GpA or *o*-azophenylgalactoside-GpA conjugates in Freund's complete adjuvant in doses of 10 μg per animal. After 10 days the guinea pigs were skin tested with 10 μg doses of 4 different antigens (Table I). The strongest delayed skin reactions were elicited in each instance with the homologous antigen. Marked cross-reactions were observed, however, with the GpA conjugates of isomeric haptens as well as with the less similar *p*-azophenylethyl alcohol-GpA conjugate. The typical carrier specificity of the delayed response may be seen in the lack of cross-reaction with the conjugate of *p*-azophenylglucoside on the unrelated protein ovalbumin. Desensitization with cross-reacting antigens eliminated the cross-reactions but did not significantly affect the homologous reaction.

Since azophenylmonosaccharides were found

TABLE II. Delayed Sensitivity Reactions to a Disaccharide-Azo-GpA Conjugate.

Sensitizing antigen	Test antigens		
	p-Azophenyl-lactoside-GpA	*p*-Azophenylethyl alcohol-GpA	*p*-Azophenyl-lactoside-Oval
	Avg skin reaction diameter (mm)		
p-Azophenyllactoside-GpA			
Not desensitized	17	11	0
Desensitized with *p*-azophenylethyl alcohol-GpA	10	0	0

to function as haptens in delayed hypersensitivity, it was of interest to do similar experiments using azophenyldisaccharides. The results obtained with *p*-azophenyllactoside-GpA conjugates are shown in Table II and are seen to be analogous in all respects to those obtained with azophenylmonosaccharide haptens.

The results described above conform to previous findings about the specificity of delayed reactions to various hapten-azo-GpA conjugates(12), but they involve a benzene ring as an integral part of each of the haptens tested. To test the haptenic capabilities of saccharide without this added nonpolar ring, protein conjugates were prepared in a different way so as to eliminate the azophenyl group. Glucuronic and galacturonic acid conjugates of GpA were prepared by the mixed anhydride method and employed for sensitization and skin testing of guinea pigs. The results (Table III) were obtained after the animals had been desensitized with isobutyl-carbamate-GpA conjugate to eliminate any contribution to the response by isobutyl groups attached to the protein during the coupling procedure. The data show (a) that these conjugates may sensitize and elicit the delayed response; (b) a strong cross-reaction between the 2 stereoisomeric monosaccharides; and (c) the typical carrier specificity, as evidenced by a lack of response to the hapten when coupled to ovalbumin as the carrier protein.

Discussion. The results described here indicate clearly that the monosaccharide and disaccharide hapten conjugate may serve quite adequately as the focus of an antigenic determinant in delayed hypersensitivity reactions. The characteristics of the response to saccharide haptens paralleled in every respect the reactions reported earlier(12,16-19) for nonionic, nonpolar haptens and for ionic haptens. Thus, these conjugates sensitized and served to elicit skin reactions of comparable intensity. They also showed the typical carrier specificity consistently observed in the delayed system, wherein conjugates of the hapten in question with unrelated protein carriers fail completely to cross-react in the skin test. Again, saccharide conjugates show the same sort of increased cross-reactivity among related haptens when attached to the same carrier protein as has been reported consistently in studies of the delayed hypersensitivity system. While Avery and Goebel(5) found conjugates of *p*-azo-phenyl-β-D-glucoside not to cross-react with the corresponding β-D-galactoside conjugates in their interaction with rabbit antibodies, the present data show that even when attached to different positions on the benzene ring, the glucoside and galactoside conjugates show

TABLE III. Delayed Sensitivity Reactions to Monosaccharide-GpA Conjugates.

Sensitizing antigens	Test antigens			
	Glucuronic acid-GpA	Galacturonic acid-GpA	Glucuronic acid-Oval	Galacturonic acid-Oval
	Avg skin reaction diameter (mm)			
Glucuronic acid-GpA	14	12	0	—
Galacturonic acid-GpA	16	14	—	0

mutual cross-reactivity in the delayed response.

Each of these points supports the conclusion that saccharide haptens do not differ in any significant respect from other ionic or nonpolar haptens examined in elicitation of delayed hypersensitivity responses. These results may be contrasted with the prediction of Karush and Eisen(11) that saccharide haptens should be ineffective as determinants in the delayed system. This latter view postulates that the delayed hypersensitivity lesion is initiated by a combination of the antigenic determinant with a hypothetical avid antibody, requiring a hapten capable of providing an extremely high interaction energy. According to this suggestion, a saccharide hapten should be incapable of interaction except with much weaker binding energies, and therefore should not meet the thermodynamic requirements of the postulated mechanism.

To test this suggestion rigorously, saccharide haptens attached directly to the carrier protein were employed, after it had been established that those attached to protein through the azophenyl linkage were active in the delayed system. This was done to exclude any contribution to the interaction energy on the part of the nonpolar phenyl group used in the original conjugates. Even with the simple monosaccharide hapten alone, however, the sensitization and elicitation of the delayed skin reactions could be accomplished without difficulty. It must therefore be concluded that saccharide haptens may serve adequately in the delayed system, and that even the relatively weak energy contributions of which the saccharide hapten is capable seem to be sufficient to mediate the response.

How, then, may one interpret the consistent inability of *purified* polysaccharides to serve as elicitors of the delayed skin reaction? A delayed hypersensitivity response has been demonstrable only in instances in which protein or peptides have been associated with the polysaccharide moiety(9,10). It will be recalled that every previous study of delayed hypersensitivity to hapten-protein conjugates has shown that both the hapten and the carrier protein to which it is attached are important to the response. The present study has demonstrated that saccharides may function adequately as haptens in this system; it would therefore appear possible that, in contrast to protein, the polysaccharide structure may not be able to fulfill all of the demonstrably important functions of antigenic carrier, the nature of which is as yet unknown.

Summary. Conjugates of monosaccharide and disaccharide haptens to protein have been found to function adequately in the induction and elicitation of delayed hypersensitivity reactions in the guinea pig. The characteristics of these delayed responses paralleled those observed with other ionic and nonpolar haptens. They exhibited the typical carrier specificity associated with the delayed reaction as well as the greater range of hapten cross-reactions than is typical of the customary anti-hapten antibody interactions. The suggestion is advanced that the absence of the delayed response to purified polysaccharides may be due to their failure to function properly as the carrier of the antigenic determinant involved in delayed reactions.

1. Heidelberger, M., MacLeod, C. M., Kaiser, S. J., Robinson, B., *J. Exp. Med.,* 1946, v83, 303.

2. Kabat, E. A., Berg, D., *Ann. N. Y. Acad. Sci.,* 1952, v55, 471.

3. Kabat, E. A., Coffin, G. S., Smith, D. J., *J. Immunol.,* 1947, v56, 377.

4. Harley, D., *J. Path. & Bact.,* 1935, v41, 491.

5. Goebel, W. F., Avery, O. T., *J. Exp. Med.,* 1929, v50, 533.

6. Goebel, W. G., *ibid.,* 1936, v64, 29.

7. Ovary, Z., Karush, G., *J. Immunol.,* 1960, v84, 409.

8. Freund, J., Bonanto, M. V., *ibid.,* 1944, v48, 325.

9. Tremaine, M. M., *Fed. Proc.,* 1963, v22, No. 2 abst. 2728, 617.

10. Barker, S. A., Cruickshank, C. N. D., Morris, J. H., Wood, S. R., *Immunology,* 1962, v5, 627.

11. Karush, F., Eisen, H. N., *Science,* 1962, v136, 1032.

12. Gell, P. G. H., Silverstein, A. M., *J. Exp. Med.,* 1962, v115, 1037.

13. Folin, O., *J. Biol. Chem.,* 1926, v67, 357.

14. Vaughan, J. R., Jr., *J. Am. Chem Soc.,* 1951, v73, 3547.

15. Harding, V. J., MacLean, R. M., *J. Biol. Chem.,* 1916, v24, 503.

16. Benacerraf, B., Gell, P. G. H., *Immunology,*

1959, v2, 53.

17. Salvin, S. B., Smith, R. R., *J. Exp. Med.*, 1960, v111, 465.

18. Benacerraf, B., Levine, B. B., *ibid.*, 1962, v115, 1023.

19. Silverstein, A. M., Gell, P. G. H., *ibid.*, 1962 v115, 1053.

Received July 26, 1963. P.S.E.B.M., 1963, v114.

Identification of Rabbit Allotypic Specificity A1 by an Isoprecipitin Produced by Immunization with Antigen-Antibody Complexes. (28649)

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The sera of guinea pigs and rabbits immunized with various antigens have beeen shown to agglutinate sheep cells sensitized with rabbit amboceptor and human Group O Rh positive erythrocytes sensitized with incomplete Rh antibodies. These antigens have included ferritin, rabbit red blood cells, *Escherichia coli, Proteus OX 19,* and autologous denatured γ-globulin, most of which antigens had been precipitated or agglutinated with their specific antisera(1-6). It has been postulated that the agglutinating factor thus produced in animals was an "anti-antibody" formed by the immunized animals against antibody globulin altered during formation of the antigen-antibody complexes, and that this might be considered comparable to the agglutination activating factor (rheumatoid factor) observed in human rheumatoid arthritis (2,4,6-8). During the course of attempts to produce rheumatoid factor-like activity in rabbits by immunization with specific immune agglutinates(9-11), a new isoprecipitin for a rabbit γ-globulin allotype was found. This report describes the method by which the isoprecipitin was obtained and characterized.

Materials and methods. Rabbits were obtained from closed colonies of the Laboratory Aids Branch, Nat. Inst. of Health. New Zealand White rabbits with allotypic specificity A4 but not A5, and Flemish Giant rabbits with both allotypic specificities A4 and A5 were used.

Type A4 donor rabbits were immunized with *Salmonella typhosa* "H" antigen* to produce high titer antiserum (1:10,000 to 1:12,000), using standard techniques(12). Anti-*S. typhosa* "H" antiserum obtained from individual rabbits was mixed with fresh *S. typhosa* "H" antigen in proportions insuring a large excess of antibody, as determined by prior titrations. 1.0 ml of a 1:10 dilution of antiserum in 0.85% NaCl was added to 1.0 ml of *S. typhosa* "H" antigen containing 1 $\times$ 10^9 organisms, and the mixtures were incubated at 50-52°C for 18 hours. The resultant agglutinates were recovered by centrifugation, washed 6 times with saline, and resuspended in saline to a final volume of 1.0 ml.

Type A4 recipient rabbits were injected with 1.0 ml of resuspended agglutinate emulsified with 1.0 ml of complete Freund's adjuvant (0.85 ml Bayol F, 0.15 ml Arlacel A, 0.4 mgm *Mycobacterium butyricum*) as follows: intradermally in the footpads, nuchal region, and back; subcutaneously in the nuchal region and back; and intramuscularly in the legs. After 6 weeks, additional injections of 2.0 ml each of saline-resuspended agglutinates (without adjuvant) were given intraperitoneally twice a week for a total of 17 booster doses, with intervals for rest and bleeding, as noted in Table I.

Sera were tested for flocculation of bentonite particles coated with human γ-globulin (B.F.T.)(9), and for anti-typhoid antibodies by standard agglutination techniques(12). Sera and their euglobulin fractions (obtained by ammonium sulphate precipitation and dialysis) were tested for agglutination of

* Kindly furnished by Col. A. S. Benenson, Walter Reed Army Inst. of Research.