

DELAYED HYPERSENSITIVITY TO HAPTEN-PROTEIN CONJUGATES

I. THE EFFECT OF CARRIER PROTEIN AND SITE OF ATTACHMENT TO HAPTEN

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Recent work has made it clear that delayed hypersensitivity reactions are immunological responses of a kind quite distinct from those dependent upon circulating antibody. The latter, presenting as they do a readily accessible molecular entity, have allowed the development of precise chemical technics for quantitative measurement, for the study of the structural and steric factors on the antigen which affect specificity, notably in work with simple chemical haptens (1), and even for an estimate of the size of the antigenic determinant involved in interaction with antibody (2, 3). Delayed hypersensitivity, presenting as a group of inflammatory reactions not susceptible to precise quantitation, and mediated by sensitized cells unamenable in general to *in vitro* studies, is less well understood. Despite the general acknowledgement that conventional circulating antibodies play no role in delayed hypersensitivity reactions (4), the mediating factor in these reactions is widely assumed to be of the nature of a conventional antibody, (but one bound intimately with the sensitized mononuclear cell and not released into the circulation), probably because the nature and mode of action of circulating antibodies at least are thought to be well understood.

One way to clarify the relationship of delayed sensitivity to antibody production, and to improve our knowledge of the basic phenomenon itself, is to study the specificity of delayed reactions. A useful model has been supplied by the recent studies of Benacerraf and Gell (5, 7), who showed that guinea pigs, sensitized with hapten-protein conjugates made from homologous (guinea pig) albumin, passed through a period of delayed hypersensitivity lasting a number of days, before the appearance of anti-hapten antibodies. They also observed some degree of "carrier specificity," i.e., that the specificity of the delayed reaction involved not only the hapten but also had an appreciable overlap onto the adjacent protein molecule. They suggested that either the size of the determinant area in the delayed sensitive cell was larger than that involved in the interaction of antigen with conventional antibody, or at least that the interactive forces were different.

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Using these observations as a point of departure the purpose of this and subsequent communications is to present a study of the immunochemical specificity of delayed reactions to hapten-protein conjugates. These results are to be contrasted with the more fully understood antigen-antibody systems, in order to compare the sizes of the respective determinants acting in the two systems, and the effect on the specificities involved of changes in the configuration of the haptenic moiety.

Materials and Methods

Protein.—Serum albumins of guinea pig (GpA), pig, rabbit, sheep, ferret, and human were all prepared in the following manner, with minor variations: serum was precipitated with ammonium sulphate to half saturation, filtered, and the albumin precipitated from the filtrate by acidification with acetic acid. After re-solution and dialysis, an equal volume of 20 per cent trichloroacetic acid was added, the precipitate was spun down, partially redissolved in 80 per cent EtOH, cleared by centrifugation, and the supernate dialyzed against running water for 24 to 48 hours. After a final centrifugation to clear, the supernate was found to be electrophoretically a fairly pure albumin slightly contaminated with a β -globulin, probably siderophilin. (In spite of this rather drastic method of purification, there was no evidence that the GpA was altered sufficiently to render it antigenic in guinea pigs; see below). Rat albumin was prepared from the urine of proteinuric rats by ammonium sulphate and acidification only.

Ovalbumin (Oval) and bovine albumin (BSA) were recrystallised commercial preparations.

Human gamma globulin (HSG) was prepared by alcohol fractionation (Cohn method 6 followed by method 9 to give a purified gamma fraction) and kindly supplied by Dr. K. Walton.

Guinea pig gamma globulin was made by precipitating three times with 16 per cent sodium sulphate, and was not pure.

The potato protein was obtained by extracting the freeze-dried sap of domestic potatoes with acetate buffer at pH 4.0, and precipitation with half-saturated $(\text{NH}_4)_2\text{SO}_4$ followed by dialysis and centrifugation.

Hapten-Protein Conjugates.—The simple chemicals used for conjugation were recrystallised commercial preparations, with the exception of *m*-aminobenzoyl-glycine, *m*-aminobenzoyl-DL-leucine, and *sym.* isophthaloyl-glycine-DL-leucine, for which we are indebted to Dr. David Pressman. Benzoic acid-*p*-isothiocyanate was prepared by adding dropwise with stirring a solution of *p*-aminobenzoic acid in dry acetone to a tenfold excess of thiophosgene in dry acetone, whereupon a precipitate formed. The suspension was stirred for an hour with heating to 60–70°C, filtered, washed with dry acetone, and then with dry petroleum ether. *p*-Isothiocyanate benzenesulfonic acid was prepared similarly, except that addition to the thiophosgene solution was effected as an acetone slurry of finely ground *p*-aminobenzenesulfonic acid.

In every instance, conjugates were prepared by the addition of a theoretical 60 haptenic groups per mole of protein. The proteins were made up as saline solutions and stirred in an ice bath. Prior to addition of the hapten, 1/10 volume of 10 per cent Na_2CO_3 was added. Aminobenzoic acid and aminobenzenesulfonic acid were converted to their diazonium derivatives with NaNO_2 in acid solution, and the product added slowly to the protein mixtures. Picryl chloride was added as an ethyl alcohol solution and *p*-nitrobenzoyl chloride as an acetone solution, the organic solvent comprising no more than 5 per cent of the final volume of the conjugation mixture. The isothiocyanate preparations were added to the protein solutions as finely divided powders, which rapidly went into solution. All conjugation mixtures were

stirred for at least 1 hour, then placed in the cold overnight, and finally dialyzed against tap water. The hapten content of the conjugates was not determined.

Sensitization of Guinea Pigs.—Suitable dilutions of the protein conjugates in isotonic saline were emulsified with an equal volume of Difco complete adjuvant (containing 1 mg of *Mycobacterium butyricum* per ml of oil). The immunizing dose (either 10 or 50 μ g) was contained in 0.2 ml of emulsion, which was distributed equally into the hind foot-pads of the guinea pig as an intradermal injection. White guinea pigs weighing 250 to 450 gm were used throughout.

Skin Tests.—At a suitable time after sensitization, usually 7 to 12 days, but occasionally longer depending upon the efficacy of the antigen concerned, the guinea pigs were tested intradermally in the flanks. The test doses were generally 0.1 ml volume, containing 10 μ g of antigen. Multiple tests were the rule, generally involving the sensitizing antigen, and one or more additional proteins bearing the same hapten. In some instances, as discussed below, tests were performed with the same carrier (GpA) bearing the same hapten, but the hapten linkage to the protein differing, as for example azobenzoate and thiocarbamidobenzoate. In all cases, it was determined at some stage of the study that the haptens themselves in the amounts used were not capable of eliciting inflammation in animals not sensitized to them.

Skin Reactions.—The time chosen for skin testing was before the appearance of Arthus sensitivity, so that observations of the test sites 2 to 4 hours later were usually negative. The tests were read and recorded at 18 to 24 hours, and the rare presence of an Arthus-like component will be noted in the Tables where present. The readings are recorded as the average diameters in millimeters of erythema presented by the lesions. While it is acknowledged that the results are not precisely quantitative and that the dose-response is ill defined, the measurements give an adequate estimate of the cross-reactivity of the various antigens compared.

Desensitization and Retesting.—On the morning following the original tests, the reactions were read, and where indicated, the animals were desensitized with the appropriate antigen, by the intraperitoneal injection of from 2 to 10 mg of antigen in the middle of the same day. After 4 to 6 hours, the animals were retested by intradermal route in a fresh area of the skin, and the reactions read the next day.

RESULTS

The Effect of the Protein Carrier on Delayed Specificity to Hapten-Protein Conjugates.—Table I illustrates the extent of cross-reactions observed in guinea pigs sensitized to 50 μ g of *m*-azobenzenesulfonate-guinea pig albumin, upon skin testing with a variety of other carrier proteins coupled with the same anionic hapten. It can be seen that in each case, the homologous antigen has given the greatest reaction. Further, conjugates of a number of serum albumins from other mammalian species have all yielded greater or lesser degrees of cross-reaction. This is in accordance with the known serological relationship which exists among the serum albumins of mammalian species, as reflected by similar types of cross-reactions observed on the part of antibodies prepared against the several serum albumins. In contrast, ovalbumin, human serum globulin, and potato protein extract have failed in every instance to provide evidence of cross-reactivity. Data are presented on individual animals, to indicate the extent of the variation observed.

The azohapten causes the antigenic determinant of the guinea pig albumin conjugate to be centered around the tyrosine, histidine, and tryptophan groups

of the protein. The use of the hapten *p*-nitrobenzoyl centers the interest primarily about the epsilon amino groups of lysine as the predominant point of attachment. The data for this system are presented in Table II. Again, it will be seen that conjugates of other mammalian serum albumins with this non-ionic hapten are able to cross-react and induce delayed sensitivity skin reactions. The completely unrelated ovalbumin, human serum globulin, and potato protein conjugates again fail to cross-react, despite their possession of the identical haptenic grouping. Guinea pig gamma globulin gives only a very minor cross-reaction due probably to contamination of the starting material with albumin. These observations stand in marked contrast to the precipitating anti-

TABLE I
Effect of Carrier Protein on Cross-Reactions of M-Azobenzene-sulphonate Conjugates

Test antigen: <i>m</i> -azobenzene-sulphonate conjugate of	Guinea pig No.						Average <i>mm</i>
	1	2	3	4	5	6	
Gp. albumin.....	12	11	11	11	11	9	10.8
Bovine ".....	8	6	8	7	9	0	6.3
Pig ".....	7	9	9	8	6	6	7.5
Sheep ".....	8	9	9	8	6	0	6.7
Rabbit ".....	9	7	9	7	0	0	5.3
Ferret ".....	5	7	4	5	0	0	4.2
Human ".....	6	4	5	7	0	0	3.7
Ovalbumin.....	0	0	0	0	0	0	0
Potato protein.....	0	0	0	0	0	0	0
Human γ -globulin.....	0	0	0	0	0	0	0

Animals sensitized with 50 μ g of *m*-azobenzene-sulphonate-GpA and tested on the 9th day after sensitization with 5 μ g of conjugates. Reaction diameters in mm.

body situation, in which it is usual to employ conjugates of completely unrelated protein carriers as the immunizing and test antigens.

Another point to be noted in Tables I and II is that not only does the homologous conjugate elicit the largest reaction, but that the order of extent of cross-reaction of the other serum albumin conjugates differs from one animal to another, possibly owing to individual variation in the response to different hapten-centered determinants. It is also noteworthy that the non-ionic hapten *p*-nitrobenzoyl shows strikingly more carrier specificity than the ionic *m*-azobenzene-sulphonate.

Desensitization of Delayed Sensitive Animals with Cross-Reacting Hapten-Protein Conjugates.—A valuable technic in working with precipitating antibody systems has proved to be the *in vitro* absorption of antisera with excess amounts of related antigens. In delayed hypersensitivity studies this technic finds its

analogy in the *in vivo* desensitization of the animal with excess injected antigen. Uhr and Pappenheimer (8) have demonstrated that guinea pigs rendered delayed hypersensitive to protein antigens became rapidly desensitized upon parenteral administration of relatively large amounts (of the order of 1 to 10 mg) of these antigens. Preliminary experiments showed that this was also generally true of guinea pigs rendered delayed hypersensitive to hapten-protein conjugates. In this and subsequent studies to be reported later, this technic has been employed extensively to desensitize animals with cross-reacting antigens,

TABLE II
Effect of Carrier Protein on Cross-Reactions of p-Nitrobenzoyl Conjugates

Test antigen: p-nitrobenzoyl conjugate of	Guinea pig No.								Average mm
	1	2	3	4	5	6	7	8	
Gp. albumin.....	20	17	11	16	12	16	25	12	16.1
Bovine "	8	5	5	6	0	6	0	0	3.7
Pig "	7	0	0	0	0	7	7	0	2.6
Sheep "	5	0	0	0	0	5	0	0	1.2
Rat "	13	0	0	0	0	5	7	0	3.1
Rabbit "	0	0	4	4	0	5	0	0	1.6
Ferret "	9	0	0	0	0	5	0	0	1.7
Human "	6	6	5	0	0	8	0	0	3.1
Ovalbumin.....	0	0	0	0	0	0	0	0	0
Potato protein.....	0	0	0	0	0	0	0	0	0
Human γ -globulin.....	0	0	0	0	0	0	0	0	0
Gp. γ -globulin.....	7	0	5	0	0	5	0	0	2.8

Animals sensitized with 25 μ g of p-nitrobenzoyl-GpA and tested on the 8th day after sensitization with 10 μ g of the various conjugates. Reaction diameters in mm.

in order to study the nature of these cross-reactions, and the heterogeneity of the specificities manifested by delayed sensitive animals.

Two groups of guinea pigs were employed, the one sensitized to 10 μ g of picryl-GpA and the other to 10 μ g of picryl-BSA. 7 days after the sensitizing injection, all animals were skin-tested both with the homologous sensitizing antigen and with the cross-reacting antigen. After reading the results the following day, the animals were divided into balanced groups, and desensitized as indicated in Table III. In each case, one group received a desensitizing dose of the cross-reacting antigen, and as a control, other groups received desensitizing doses of homologous antigen, or no desensitization at all. Since the animals sensitized to picryl-BSA might be expected to become delayed sensitive both to non-coupled determinant areas in the BSA molecule, as well as to determinants possessing the picryl group, a fourth group in this set were desensitized with a mixture containing both picryl-GpA and uncoupled BSA.

It will be seen from Table III that where no desensitization was performed, the second set of skin tests approximated in intensity to the reactions given by the original tests. The

very slight diminution in average intensity observed is in all probability ascribable to the slight desensitizing effect of the first test performed the day before, as discussed by Uhr and Pappenheimer (8). Where the homologous sensitizing antigen was employed for desensitization, the animals, with one minor exception, were completely desensitized both to the homologous and to the heterologous antigen, as would be expected.

Quite different are the results of desensitization with the heterologous cross-reacting antigens. In animals delayed sensitive to picryl-BSA, desensitization with picryl-GpA served to eliminate completely all trace of cross-reaction, while significantly reducing the degree of the homologous reaction. In animals delayed sensitive to picryl-GpA, desensitization with picryl-BSA again almost com-

TABLE III

The Effect of Homologous and Heterologous Desensitization on the Homologous and Cross-Reacting Skin Tests

The number of positively reacting animals in each sub-group is recorded, followed in brackets by the mean reaction diameter of the *positive* animals.

Sensitizing antigen	No. of animals	Initial skin test				Desensitizing dose	Final skin test			
		With picryl-GpA		With picryl-BSA			With picryl-GpA		With picryl-BSA	
Picryl-GpA	6	6/6	(17)	6/6	(12)	5, Picryl-BSA	6/6	(16)	2/6	(8)
	3	3/3	(14)	1/3	(10)	10, Picryl-GpA	1/3	(7)	0/3	
	6	6/6	(17)	6/6	(13)	None	6/6	(16)	6/6	(12)
Picryl-BSA	6	5/6	(9)	6/6	(15)	5, Picryl-GpA	0/6		6/6	(11)
	5	4/5	(11)	5/5	(16)	5, " "				
						plus 5, picryl-BSA	0/5		5/5	(10)
	3	2/3	(12)	3/3	(15)	10, Picryl-BSA	0/3		0/3	
	5	4/5	(10)	5/5	(17)	None	4/5	(9)	5/5	(15)

pletely eliminated the response to this cross-reacting antigen. In this case, however, the homologous reaction to picryl-GpA was essentially unaffected.

It is, finally, of some interest to point out that the addition of normal BSA to the desensitizing dose of picryl-GpA in animals sensitized to picryl-BSA made little difference in the extent of residual reaction to the homologous antigen. This suggests that the delayed sensitivity of the animal was primarily directed against areas on the BSA molecule to which picryl groups were attached, and that little or no sensitivity was directed against normal, unaltered BSA determinants. Since the picryl conjugates employed in this study were all rather highly substituted, it is perhaps not surprising that no uncoupled areas on the conjugate molecule were available to react as determinants.

The Effect of the Site of Attachment of the Hapten upon Delayed Specificity to Hapten-Protein Conjugates.—Whereas it has been common in work with anti-

hapten antibodies to employ different carrier proteins as test antigens, the carrier seems to play a major role in the delayed hypersensitivity reaction. This is made clear by the previous observations of Gell and Benacerraf (7), Benacerraf and Levine (9), and by the data presented above on the lack of cross-reaction of unrelated proteins coupled to the same haptenic determinant by the same chemical linkage. If indeed both the hapten and the adjacent area on the carrier protein are each necessary to define a determinant with respect to delayed hypersensitivity reactions, then it might be predicted that the same hap-

TABLE IV
The Effect of Mode of Linkage of the Hapten on the Specificity of Delayed Hypersensitivity Skin Reactions in Conjugate-Sensitized Guinea Pigs

Immunizing antigen: guinea pig albumin conjugate of	Skin test antigen: guinea pig albumin conjugate of					
	<i>p</i> -NCS-benzene sulfonate	<i>p</i> -Azo-benzene sulfonate	<i>p</i> -NCS-benzoate	<i>p</i> -Azo-benzoate	<i>p</i> -NO ₂ -benzoyl	<i>p</i> -NO ₂ -benzeneazo
	<i>mm</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>	<i>mm</i>
Benzenesulfonate- <i>p</i> -NCS*	15	0	—	—	—	—
<i>p</i> -Azobenzene sulfonate*	0	15	—	—	—	—
<i>p</i> -NCS-benzoate	—	—	16	0	—	—
<i>p</i> -Azobenzoate	—	—	0	9	—	—
<i>p</i> -NO ₂ -benzoyl	—	—	—	—	11	0
<i>p</i> -NO ₂ -benzeneazo	—	—	—	—	0	12

* Cross-desensitization between these antigens showed no effect, in doses sufficient to abolish an homologous reaction.

ten attached at two different points on the same protein might serve to define two distinct antigenic determinants in the delayed system.

In order to achieve this situation, conjugates of GpA were made with several different simple haptens. These were *p*-benzoate, benzene-*p*-sulfonate, and *p*-nitrophenyl. In each instance, two derivatives of each hapten were used, the one being a diazonium salt which couples primarily to tyrosine, tryptophan and histidine, and the other predominantly an amino-coupling agent, either the isothiocyanate or the acid chloride. One group of animals were sensitized to each of the haptens of a pair, and then tested both with the homologously linked hapten conjugate (used for sensitization), and with the conjugate of the same hapten and protein, but linked to one another differently. 10 μ g of conjugate was used for sensitization in the *p*-nitrophenyl and benzenesulfonic acid systems; 50 μ g of conjugate was used for the benzoate sensitizations. In every case, the skin test doses were of 10 μ g amounts. These data are presented in table IV.

It is immediately apparent from this Table that the specificity of the delayed hypersensitivity reaction is such that it can distinguish the type of linkage by which a given hapten is attached to a given protein carrier. In no instance was a cross-reaction observed in any of the hapten-protein pairs, among which

the only difference was the site of attachment of the hapten on the carrier protein surface. Cross-desensitization was attempted on the *p*-NCS-benzene-sulfonate/*p*-azobenzenesulfonate pair and was entirely ineffective in doses sufficient to abolish completely the homologous reaction.

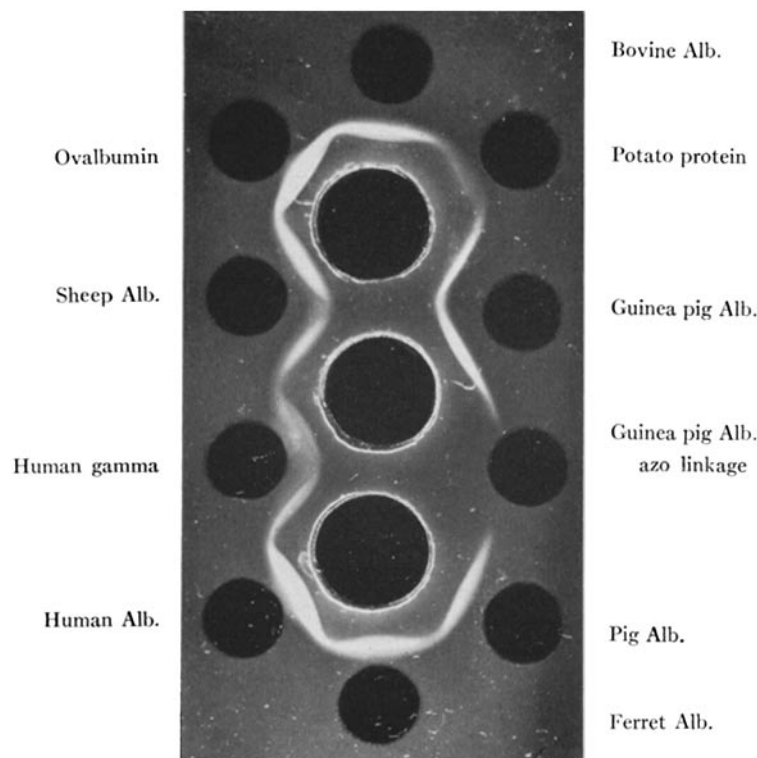


FIG. 1. Reactions of anti-*p*-nitrobenzoyl guinea pig antiserum with various *p*-nitrobenzoyl proteins (Alb. = albumin) and with *p*-nitrophenylazo guinea pig albumin.

This result cannot be taken as an argument for a clearcut difference between delayed specificity and antibody specificity; we present in Fig. 1 an analogous situation found among precipitating antibodies, when cross-tested with the same hapten linked differently to the same protein. In this case, delayed hypersensitive guinea pigs were allowed to go on to antibody production, and the resultant precipitin tested by gel diffusion both with the homologously linked conjugates and with the same hapten linked differently to the same protein. It will be seen that the antibody specificity in this instance is not solely determined by the simple hapten, but appears also to involve at least the amino acid to which the hapten is attached, in the one case predominantly lysine, in

the other predominantly tyrosine. The presence of a cross-reaction between this antibody and the homologously linked hapten on an ovalbumin carrier shows that the precipitin system is not subject to the same degree of carrier specificity as the delayed system discussed above.

A Preliminary Observation on the Determinant Size Effective in Delayed Sensitivity Reactions.—In their attempt to define the maximum size of the antigenic determinant effective in antigen-antibody reactions, Landsteiner and Van der Scheer (10) employed haptens possessing two distinct antigenic specificities. These investigators concluded that rabbits injected with protein conjugates of *sym.*-azoisophthaloyl-glycine-DL-leucine responded with little or no antibody production against the entire haptenic determinant. Rather, the animals were

TABLE V

Delayed Hypersensitivity in Animals Sensitized to GpA-Azo-Isophthaloyl-Glycine-dl-Leucine Effect of Desensitization with a Mixture of GpA-m-Azobenzoyl-Glycine (2 mg) and GpA-m-Azobenzoyl-DL-Leucine (2 mg) on Delayed Skin Reactions

Test antigen: 10 μ g GpA conjugate of	Guinea pig No.					
	1	2	3	4	5	6
Glycine-leucine: difunctional hapten.....	16/10	16/16	15/8	16/0	15/0	17/19
Leucine hapten.....	7/0	11/0	10/0	12/0	12/0	12/13
Glycine hapten.....	11/0	10/0	9/0	10/0	12/0	13/13

Animal No. 6 was an un-desensitized control: in each instance the first figure is the reaction diameter before desensitization and the second that after desensitization with the mixture of the two monofunctional haptens.

found to make antibodies specific for either the one group (*m*-azobenzoyl-glycine) or for the other (*m*-azobenzoyl-DL-leucine).

In order to compare these results with the delayed hypersensitivity mechanism guinea pigs were sensitized with 50 μ g of GpA coupled to the difunctional glycine-leucine compound through the azo linkage. After 15 days, the animals were skin-tested with 10 μ g doses of the homologous difunctional antigen, and also with conjugates of GpA with the monofunctional benzoyl-glycine and benzoyl-DL-leucine compounds. The following day, the reactions were read, and the animals were then desensitized with 2 mg each of the azo-benzoyl-glycine and azo-benzoyl-DL-leucine conjugates. 5 hours later, the same set of skin tests were performed.

The results of this preliminary experiment are recorded in Table V. The control undesensitized animal showed no change in its reactivity. The results among the desensitized animals used are somewhat disparate. Two of the animals were completely desensitized, not only to the cross-reacting monofunctional compounds, but also to the homologous difunctional antigen. Significantly, however, three animals showed a complete disappearance of the cross-reaction

to the monofunctional antigens, and a reduced, but quite significant residual reaction to the homologous difunctional antigen used for the original sensitization, suggesting that they had in part become sensitized by, and could respond to, the complete difunctional haptenic determinant.

DISCUSSION

In contrast to the relative ease with which antibodies with a predominant hapten specificity may be elicited by hapten-protein conjugates, the development of a strictly hapten-oriented delayed hypersensitivity is appreciably more difficult to demonstrate (11). Thus, animals rendered delayed hypersensitive to modest doses of picryl-BSG gave delayed skin test reactions to the homologous antigen, but not to picryl-Oval. Only after the animals had progressed to antibody production would they give anaphylactic responses indiscriminately to either conjugate. This latter characteristic of antibodies is the basis for the common practice (1) of preparing anti-hapten antibodies with one carrier protein and testing the anti-hapten specificity with a conjugate of an unrelated protein. Based on such data, it was proposed (5) that the specificity of the delayed hypersensitivity reaction may involve an appreciable overlap beyond the haptenic moiety onto the surrounding surface of the carrier protein.

The data presented in this communication verify and extend the observations referred to above. In Tables I and II are recorded the data on the extent of cross-reaction observed with a variety of hapten-protein conjugates in guinea pigs sensitized to GpA conjugates. The results were similar, both with the anionic *m*-azobenzenesulfonate system, which couples to protein primarily onto tyrosine and histidine groups, and with the non-ionic *p*-nitrobenzoyl hapten, a primary amine-coupling agent. No cross-reactions were observed with conjugates of the completely unrelated proteins HSG, ovalbumin, or potato protein. Cross-reactions were found, however, to all of the more-or-less related mammalian serum albumins employed as protein carriers of the hapten involved. Since the several mammalian serum albumins have been recognized as being serologically related for phylogenetic reasons, the cross-reactions observed are not unexpected. The failure of cross-reaction among the other conjugates, despite the fact that they bear the identical hapten on the same amino acids and are efficient precipitating antigens with guinea pig anti-hapten sera (*cf.* Fig. 1) seems to support the notion that in animals sensitized and tested with the present dosage schedules, there exists little or no specificity directed solely against the haptenic moiety. Rather, the carrier protein itself is seen to constitute a necessary portion of the antigenic determinant which interacts with the mononuclear cell carriers of delayed sensitivity to result in the delayed sensitivity lesion.

The extent of cross-reaction between conjugates of closely related serum albumins is brought out in greater detail in Table III. The antigens employed

here were picryl-GpA and picryl-BSA. The extent of cross-reaction in this system is seen to be considerable. It is unclear at present why the picryl-BSA antigen should have reacted relatively more strongly in picryl-GpA-sensitized animals than picryl-GpA in picryl-BSA-sensitized animals.

By way of further pointing up the nature of the specificity involved in the delayed sensitivity reaction to hapten-protein conjugates, and as an indication that both the hapten and its immediately adjacent carrier protein surface are each required to elicit the reaction, haptens were coupled to a given carrier protein by two different chemical routes. Each of the hapten-protein conjugate pairs were identical both with respect to the hapten used and the protein employed, differing only in the point of attachment onto the protein's surface. In the one case, attachment was primarily to lysine groups; in the other, to tyrosine, tryptophan, and histidine sites (Table IV). In no instance was a cross-reaction observed. These data suggest that not only are the haptenic portion of the determinant and the carrier protein portion both necessary to elicit delayed hypersensitivity, but that they must be present together, and in the same relationship to one another as existed in the molecule used for sensitization. Both moieties are necessary, but neither member alone suffices to affect the delayed hypersensitive animal. This observation is further borne out by the desensitization data of Table III. Here it may be seen that guinea pigs made delayed hypersensitive to picryl-BSA could not be completely desensitized with a mixture of BSA and picryl-GpA, which contained an excess of both the carrier protein and the hapten. Only those determinants shared by picryl-GpA seem to have been suppressed.

Since anti-hapten antibodies appear often to be regarded as possessing a purely hapten-specific action, it might appear that the data discussed above on the effect of the haptenic linkage to carrier protein in delayed reactions represent a striking difference between delayed and antibody specificity. That this is not always true is shown by the observation that anti-*p*-nitro-benzoyl-GpA antibodies raised in the guinea pig will not cross-react with *p*-nitrobenzeneazo-GpA, but will react with *p*-nitrobenzoyl-ovalbumin, etc. (Fig. 1). This suggests that even precipitating antibodies may require for their specificity not only the hapten itself, but also the immediately adjacent amino acid to which the hapten is attached. A similar observation was made by Mutsaers and Gregoire (12), who showed that with at least some rabbit anti-hapten sera, no precipitin cross-reaction could be observed when the mode of linkage of hapten to protein was changed. It is of interest, however, that several of their antisera did show cross-reactions independent of the nature of the haptenic linkage to the carrier. This contrasts with the complete absence of such cross-reactions in delayed hypersensitive animals described above. The effect of hapten linkage on antibody specificity is being further investigated, and will be discussed at greater length elsewhere. As for the carrier protein, Haurowitz (13), Haurowitz and

Schwerin (13), and Francis *et al.* (14) have shown that with some anti-hapten antisera at least, there exists a small proportion of the antibody which appears to be truly carrier-specific. Nevertheless, the invariable presence of cross-reactivity independent of the protein carrier stands in sharp contrast to the case with which carrier specificity can be demonstrated in the delayed situation.

What, then, may be said about the size of the antigenic determinant functionally active in the delayed hypersensitivity reaction?

At present it can be concluded that at least at the stage of development of delayed hypersensitivity which we have investigated the reaction requires an apparently larger determinant area than does the circulating antibody in the rabbit. It is known, for instance, that anti-phenylarsonate antibody will interact with and bind so simple and small a chemical as phenylarsonate (14), and that guinea pigs may be sent into anaphylactic shock with a simple polyvalent hapten (15). While the work of Leskowitz and Kabat has indicated a maximum effective size of the antigenic determinant with respect to precipitin formation as that of tri- to hexasaccharide (2), the mono- or disaccharide seems to present a large enough determinant area so that the antigen-antibody reaction may proceed, the terminal hexose contributing at least 40 per cent of the binding energy. Goebel *et al.* (16) found moreover that with rabbit antibody to the *p*-aminophenyl glycosides of di-hexoses, inhibition was almost as strong with the *p*-aminophenyl glycoside of the terminal sugar as with that of the terminal di-hexose. It is significant that before the appearance of antibody, the guinea pig delayed sensitive to a hapten-GpA conjugate will not support a cross-reaction of the same hapten coupled to ovalbumin. After antibody production has begun, however, this animal will give Arthus reactions indiscriminately to both the homologous and the heterologous conjugates (9). In the former instance, carrier specificity is being manifested; in the latter, it seems to be lacking.

In an effort to ascertain the maximum determinant size to which the antibody-producing mechanism could respond, Landsteiner and Van der Scheer (10) employed for immunization a conjugate of *sym.*-aminoisophthaloyl-glycine-DL-leucine. They concluded, based upon absorption studies, that little or no antibody was formed against the total hapten. Rather, they found antibodies against either the glycine or the leucine moieties. We have observed in a preliminary experiment (Table V) that guinea pigs rendered delayed hypersensitive to a conjugate of this difunctional compound could often not be completely desensitized with conjugates of the monofunctional constituents. It thus appears that the delayed hypersensitivity mechanism may result in a specificity capable of encompassing the full size of the difunctional hapten used.

The possibility that a fraction of the delayed sensitive cells in an animal may be able to act free of the restrictions of carrier protein specificity, (*i.e.*, may be hapten-specific) is suggested by the data of Benacerraf and Gell (2). Cross-reactions independent of the nature of the protein carrier were obtained by these workers, however, only with the use of heroic measures, involving sensitizations with 100 to 1,000 times the ordinary doses of antigen, and with the use of skin test doses very much larger than ordinarily employed. In the normal course

of events, with small doses of antigen such as employed in the present study, it may reasonably be concluded that few if any cells participating in the delayed reaction are able to respond to haptens alone, without the intervention of carrier protein specificity.

In the present discussion, as in earlier ones, the differences in specificity of the delayed hypersensitivity reaction and the reactions of circulating antibody have been expressed in terms of apparent differences in the size of the determinant required to initiate a response. It is appreciated that reference to a *functionally* larger determinant does not necessarily imply a real difference in total area participating in the immunological interaction. It is unlikely that the energy of interaction of antigen with the receptor on the delayed hypersensitive cell will be found ultimately to be of an order different from those involved in other similar biological reactions, such as between antigen and antibody. Among the possibilities which present themselves to explain the specificity differences between delayed and antibody reactions are:

1. That roughly the same interaction energy is, in the delayed system, distributed over a greater determinant area, with the haptenic end of the determinant not itself sharing enough of the total binding energy to initiate a reaction in the delayed sensitive cell.

2. That the determinant sizes in the two situations are actually of the same order of magnitude, but that for some reason the distribution of interaction energy differs. If the interaction energy were distributed more-or-less equally over the determinant, rather than being fairly concentrated within a small area, as in the antibody situation, then the share of the total interaction energy contributed by the hapten in the delayed system might be insufficient to trigger the reaction.

3. That the interaction of antigen with the delayed sensitive cell may be a multi-stage reaction, involving at one step the terminal (hapten) portion of the determinant, and at another stage, the carrier protein moiety.

None of these arguments excludes the possibility that some, at least, of the antibodies formed in the early stage of production may show similar specificity characteristics to those of the delayed reaction (a point which is under investigation) nor, though this seems less likely, that during the later stages of antibody production, any persisting delayed sensitive cells may reflect the narrowed kind of specificity of conventional antibodies.

If indeed the delayed mechanism responds in general only to the combination of hapten with its surrounding carrier area, then a highly substituted conjugate may present almost as many different determinants as there are haptenic groupings on the molecule, since there is probably little repetition of amino acid sequences in proper orientation to one another in the proteins generally employed. If this be so, sensitization with a conjugate of even the most highly purified protein will result in the production by the recipient animal of a group of sensitive cells specific for each determinant present, *i.e.*, there will be en-

countered an antigen-induced heterogeneity of specificities among a very large number of clones of sensitive cells. We would term this type of heterogeneity "heteromorphic," to indicate that it is directed against determinants of different form. In the response of animals to hapten-protein conjugates, a heterogeneity of interaction energies has been described among the antibodies produced, such that the less well "fitting" antibodies will cross-react against haptens of slightly differing structures. In contrast to the above defined *hetero*-morphic heterogeneity, this latter might be termed "*isomorphic*" heterogeneity, to indicate a diversity of response to the same form of inducing antigen.

Another consequence of the inability of the hapten alone to elicit the delayed reaction is the relatively less dominant role which it plays in the total determinant, in comparison with the prominent contribution by the hapten to antibody-hapten interactions. In a situation wherein the hapten plays such a lesser role, it might be expected that the immune system (delayed) would manifest a wide range of cross-reactions among related haptenic structures. This has indeed been found to be the case, and is the subject of the following paper.

SUMMARY

Further data have been presented showing that the specificity of the delayed hypersensitivity reaction in the guinea pig to hapten-protein conjugates involves to a considerable degree a contribution by the protein carrier. The carrier contribution is such that sensitization to guinea pig albumin-*m*-azobenzene-sulfonate, for example, does not result in cross-reaction with conjugates of the same hapten with unrelated proteins such as ovalbumin or human gamma globulin, nor were cross-reactions observed between conjugates prepared with the same hapten, coupled to the same protein, but by two different chemical routes, such that the point of attachment of the hapten to the protein differed. It thus appears that in this system both hapten and carrier protein are necessary, but that neither alone is in general sufficient to stimulate the delayed sensitive cell.

Desensitization experiments with cross-reacting hapten-protein conjugates have suggested the presence of a multiplicity of antigenic determinants participating in the elicitation of the delayed lesion, and of a concomitant development of a heterogeneity of specificities in the population of delayed sensitive cells in the sensitized animal.

The data are discussed in terms of the apparent requirement of the delayed sensitivity mechanism for a larger functional antigenic determinant than that required for interaction with circulating antibodies. Some possible explanations for this difference, and some of its consequences, are discussed.

BIBLIOGRAPHY

1. Landsteiner, K., *The Specificity of Serological Reactions*, Cambridge, Harvard University Press, 2nd edition, 1945.

2. Kabat, E. A., and Leskowitz, S., Immunochemical studies on blood group substances. XVII. Structural units involved in blood group A and B specificity, *J. Am. Chem. Soc.*, 1955, **77**, 5159.
3. Karush, F., Interaction of purified anti-beta-lactoside antibody with haptens, *J. Am. Chem. Soc.*, 1957, **79**, 3380.
4. Pappenheimer, A. M., Jr., Scharff, M., and Uhr, J. W., in *Mechanisms of Hypersensitivity*, (J. H. Shaffer, editor), Boston, Little, Brown and Co., 1959, 435-450.
5. Benacerraf, B., and Gell, P. G. H., Studies on hypersensitivity. III. The relation between delayed reactivity to the picryl group of conjugates and contact sensitivity, *Immunology*, 1959, **2**, 219.
6. Salvin, S. B., and Smith, R. F., Delayed hypersensitivity and the anamnestic response, *J. Immunol.*, 1960, **84**, 449.
7. Gell, P. G. H., and Benacerraf, B., Studies on Hypersensitivity. IV. The relationship between contact and delayed sensitivity, *J. Exp. Med.*, 1961, **113**, 571.
8. Uhr, J. W., and Pappenheimer, A. M., Jr., Delayed Hypersensitivity. III. Specific desensitization of guinea pigs sensitized to protein antigens, *J. Exp. Med.*, 1958, **108**, 891.
9. Benacerraf, B., and Levine, B. B., Immunological specificity of delayed and immediate hypersensitivity reactions, *J. Exp. Med.*, 1962, **115**, 1023.
10. Landsteiner, K., and Van der Scheer, J., On cross-reactions of immune sera to azoproteins. II. Antigens with azocomponents containing two determinant groups, *J. Exp. Med.*, 1938, **67**, 709.
11. Benacerraf, B., and Gell, P. G. H., Studies on hypersensitivity. I. Delayed and Arthus-type skin reactivity to protein conjugates in guinea pigs, *Immunology*, 1959, **2**, 53.
12. Mutsaers, W., and Gregoire, P. E., Comparaison entre les caractères antigeniques des matières protéique conjuguées a des noyaux aromatiques par des liaisons uréide ou azoïque, *Compt. rend. Soc. biol.*, 1936, **123**, 144.
- 13.(a) Haurowitz, F., Ueber die Bindung zwischen Antigen und präzipitierendem Antikörper, *Z. phys. Chem.*, 1937, **245**, 23. (b) Haurowitz, F., and Schwerin, P., Specificity of antibodies to antigens containing two different determinant groups, *J. Immunol.*, 1943, **47**, 111.
14. Francis, G. E., Mulligan, W., and Wormald, A., Use of radioactive isotopes in immunological investigations, reactions of antisera to antigens containing multiple determinant groups, *Biochem. J.*, 1955, **60**, 118, 363, 370.
15. Pressman, D., Brown, D. H., and Pauling, L., The serological properties of simple substances. IV. Hapten inhibition of precipitation of antibodies and polyhaptenic simple substances, *J. Am. Chem. Soc.*, 1942, **64**, 3015.
16. Goebel, W. F., Avery, O. T., and Babers, F. H., Chemo-immunological studies on conjugated carbohydrate proteins. IX. The specificity of antigens prepared by combining the *p*-aminophenol glycosides of disaccharides with protein, *J. Exp. Med.*, 1934, **60**, 599.