

Preparation and Immunological Properties of Procaine-Protein Conjugates

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Procaine was conjugated to BSA and rat and rabbit Gf using the carbodiimide method and ^{14}C -procaine as a tracer. The composition of the conjugates could be varied depending on the time of incubation and the concentration of procaine in the reaction mixture. Procaine-BSA conjugates were soluble in water or saline. However, procaine conjugates to rat or rabbit Gf were not readily soluble in saline. These conjugates were good for immunization purposes, but it was cumbersome to work with them when clear solutions were needed, as in the immunochemical procedures used in this study. The immunological properties of the conjugates were studied in rats and rabbits. Rats responded with production of IgGa and precipitating antibodies to the procaine group, but IgE antibodies to the immunogen could not be detected. Furthermore, precipitating antibodies towards the procaine group were raised in rabbits. When BSA was the protein carrier, antibodies to the carrier molecule were also detected in both rats and rabbits. The conjugates of procaine to rat or rabbit Gf did not elicit antibody response to the carrier molecule when used in the homologous species. Hapten inhibition studies suggested that, in the rabbit, antibodies were also produced with specificity directed towards the molecular configuration of the hapten-carrier bond.

Key words: antibodies to procaine; drug allergy; procaine; procaine-protein conjugates.

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Recently we have been interested in studying whether drugs which have been implicated clinically as causing allergic reactions in man are immunogenic in animals and particularly whether they can induce the production of anaphylactic antibodies to drug determinants. In this respect our previous studies with penicillin (8, 9, 17) have indicated that rats responded with anaphylactic antibody production to the penicilloyl group when fed with penicillin or immunized with penicillin or penicilloyl-protein conjugates. Both IgE and IgGa type antibodies were detected. If anaphylactic antibodies in animals are produced to drugs which are associated with allergic reactions in man and only to these drugs, then a simple screening model could be devised to determine the sensitizing potential of drugs in man.

Procaine and local anesthetics generally have been implicated clinically as causing allergic reactions in man (3, 4, 13). The frequency and severity of the reactions to procaine are not, however, as high as with penicillin. It was of interest to determine if procaine could elicit an antibody response in experimental animals and how this response would compare to that obtained previously with penicillin (8, 17). Furthermore, therapeutic monitoring of drugs has become of major importance and has stimulated the development of new assay systems among which radioimmunoassays play a predominant role as they are sensitive, specific and suitable for automation. As we were investigating the immunologic response of animals to the procaine group, the production of precipitating antibodies to this group was also studied with the pur-

pose of finding whether rabbits could respond with sufficiently high titers of antibodies to procaine suitable for the development of radioimmunoassay tests.

Most drugs having a molecular weight of less than 1000 are non-immunogenic by themselves. However, the binding of a small molecule, hapten, to a large carrier protein, permits the hapten to gain access to the antibody-forming cells (7). Thus our initial efforts were directed towards conjugating procaine to proteins *in vitro*. The immunogenicity of the procaine-protein conjugates was then studied in rats and rabbits. This communication is a report of our findings.

MATERIAL AND METHODS

Animals. Albino Wistar male rats (Woodlyn Farms, Guelph, Ontario, Canada) weighing 200–250 g were used for immunization, as a source of normal rat serum (NRS), and for performing passive cutaneous anaphylaxis (PCA) tests. New Zealand White rabbits (Laboratory Animal Farms, Rockland, Ontario, Canada) weighing 2.5–3.0 kg were used for immunization and as a source of normal rabbit serum.

Chemicals and biochemicals. Procaine (*p*-aminobenzoyldiethylaminoethanol) hydrochloride was purchased from BDH Chemicals (Toronto, Canada); ^{14}C -procaine (carboxyl- ^{14}C -aminobenzoyldiethylaminoethanol) hydrochloride from New England Nuclear (Dorval, Quebec, Canada); 1-ethyl-3(3-dimethylamino-propyl)-carbodiimide (EDC) and bovine serum albumin (BSA) from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); *o*-phthaldialdehyde from Calbiochem (San Diego, Calif., U.S.A.); pertussis vaccine from the Connaught Laboratories, Ltd. (Willowdale, Ont., Canada); complete and incomplete Freund's adjuvant and noble agar from Difco Laboratories (Detroit, Mich., U.S.A.); UM2 and UM10 Diaflo ultrafiltration membranes from Amicon Corp., (Lexington, Mass., U.S.A.); and Sephadex G-25, coarse, from Pharmacia Fine Chemicals (Uppsala, Sweden). Buffers were made using reagent grade chemicals.

Conjugation of procaine to proteins. Procaine was coupled to proteins using as a coupling agent the water soluble carbodiimides (6). The following will serve as an example of the procedure used: to 25 mg of BSA in 5 ml water were added 400 mg procaine and 500 μl ^{14}C -procaine (specific activity: 2.58 Ci/mm). The pH was adjusted to between 5.4–5.6. Then 25 mg EDC were added and the mixture was incubated for 48 h. At the end of the incubation period the mixture was passed through a Sephadex G-25 column (2.5 $\times$ 100 cm) equilibrated with water. The first protein peak was collected, concentrated using UM2 Diaflo ultrafiltration membranes, dialyzed against water and lyophilized. Protein was determined employing the Lowry method (10) using crystalline BSA as standard. Radioactivity measurements were taken on 1 mg of the conjugate dissolved in 200 μl of water. Bray's solution (1), 10 ml, was added and the sample was counted in a Packard Liquid Scintillation counter. From these measurements and the known amount of radioactivity added along with the procaine concentration in the incubation mixture, the composition of the conjugates was calculated. To obtain conjugates of different composition, the amount of procaine (from 40 to 400 mg) and the time of incubation (from 24 to 72 h) were varied. Procaine was similarly conjugated to the globulin fraction (Gf) of rat or rabbit serum obtained by 50% ammonium sulfate precipitation of the corresponding serum. Water was replaced by saline in all steps of conjugation and isolation of these conjugates.

Preparation of control proteins. To 25 mg of protein in 5 ml water or saline, pH 5.4 to 5.6, 25 mg of EDC were added. The mixture was allowed to stand at room temperature for 24 h. The resulting protein was subjected to the same purification procedure as used for the conjugates.

Immunization procedure. a) Rat IgE antibody production. Groups of 12 rats were immunized by i.p. injection of 1 mg of the antigen (procaine-protein conjugates) plus 7×10^9 killed *Bordetella pertussis* organisms. Twelve to 14 days later the animals were bled and sera collected. The sera were kept refrigerated for initial testing then

divided into 1-ml samples and stored at -70°C . b) Rat IgGa antibody production. Groups of 12 rats were immunized in the foot pads and i.m. with 1 mg of the antigen in complete Freund's adjuvant. Six weeks later they were reimmunized as above and were bled on the 8th week. c) Rabbit immunization. Rabbits were immunized by monthly injections of 1 mg of the antigen in complete (initial immunization) and incomplete (subsequent immunizations) Freund's adjuvant. Initial injections were made in multiple sites in the foot pads and i.m. in several sites. Rabbits were bled at monthly intervals.

Absorption of antisera: To 1 ml aliquots of undiluted antisera was added BSA at 1 mg increment until complete absorption was achieved. After incubation at room temperature for 4 h and at 4°C for 20 h the mixture was centrifuged at 2,000 g for 30 min at 4°C . The supernatants were then tested by immunodiffusion for completion of absorption which was achieved in most cases by one absorption. The antiserum thus absorbed no longer reacted with BSA and is referred to as 'absorbed' serum.

Passive cutaneous anaphylaxis (PCA) assays. PCA tests were performed according to the method described by Ovary (15). The 4-h and 24-h PCA reaction to assess IgGa (12) and IgE (20) antibodies respectively were employed. Antigens were used at a concentration of 1 mg/ml. Two to four recipients were used for each PCA assay.

Double diffusion experiments. Double diffusion experiments (14) were performed in 1.0% noble agar in borate saline buffer using glass slides (2.5×7.6 cm). In some cases, the slides were washed for 24 h in saline containing procaine (10 mg/ml; 100 ml with one change) before drying and staining. Such treatment was expected to dissolve bands formed as a result of reaction between procaine determinants and anti-procaine antibodies but having no effect on bands due to antibody-antigen reactions of other specificities. Reactions were allowed to proceed at room temperature and precipitin lines were observed within 24 h. The plates were washed for

24 h with saline, dried and stained with a solution of Amido Schwarz 10B. Wells were filled with 10 μl of antigen solution at the concentrations indicated in the text.

RESULTS

Conjugation of procaine to proteins. Procaine has been conjugated to either BSA, rat Gf or rabbit Gf. Table 1 shows the hapten content of these conjugates as determined from protein and radioactivity measurements. The conjugates of procaine to BSA were soluble in either water or saline. However, the conjugates of 'procaine to rat or rabbit Gf were not readily soluble in saline. They were good immunogens (see immunogenicity of procaine-protein conjugates) but not convenient to work with when clear solutions were needed as in immunodiffusion studies.

Table 1
Composition of procaine-protein conjugates

1. Procaine₇-BSA*
2. Procaine₁₈-BSA
3. Procaine₃₀-BSA
4. Procaine₁₉-Rat Gf
5. Procaine₅₄-Rat Gf
6. Procaine₂₉-Rabbit Gf

*Subscript refers to moles of procaine per mole of carrier protein.

Immunogenicity of procaine-protein conjugates in rats. Procaine-BSA conjugates (7, 18 and 30 mol procaine/mol BSA) and procaine₁₉-rat Gf were hardly immunogenic in rats with respect to IgE antibody production. Sera from only two out of 24 animals immunized with the former antigen gave a very weak positive 24-h PCA reaction on challenge with procaine-BSA. None of the animals immunized with the latter antigen responded with IgE antibody production. Rats immunized with either procaine-BSA (18 and 30 mol procaine/mol BSA) or procaine₁₉-rat Gf responded with IgGa antibody production. Table 2 shows 4-h PCA results using rat anti-procaine-BSA serum with procaine-BSA, BSA or carbodiimide-treated BSA as challenging antigens.

Table 2
Specificity of rat anti-procaine-BSA sera as determined by
4-h PCA tests

Rat antisera	Reactions in mm of diameter to:		
	BSA	Procaine-BSA	Carbodiimide treated BSA
Anti-procaine-BSA	20×20	30×30	ND**
'Absorbed' anti-procaine-BSA	N*	25×25	N
Normal rat serum	N	N	ND

*N, Negative
**ND, Not done

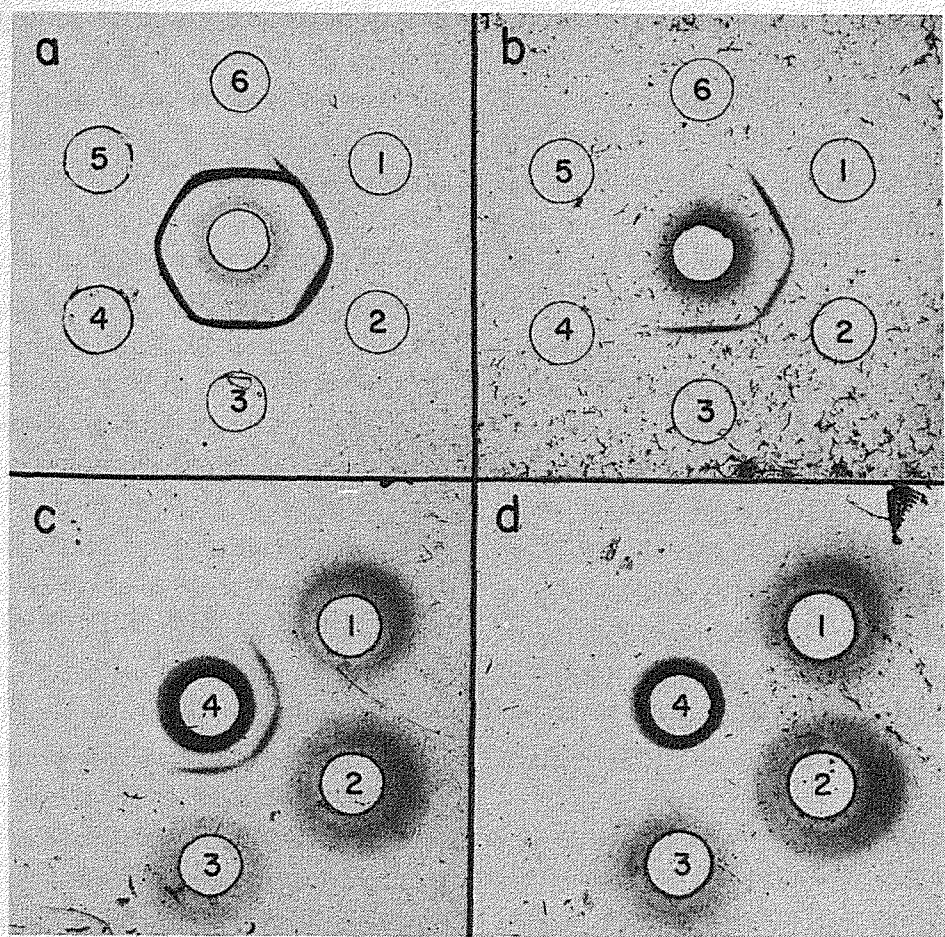


Fig. 1. Double immunodiffusion analysis of rat anti-procaine-protein sera.
1a. Center well: anti-procaine-BSA serum.
Wells 1, 2, 3: procaine-BSA (1 mg/ml in wells 1 and 3; 0.5 mg/ml in well 2).
Wells 4, 5, 6: BSA (1 mg/ml in wells 4 and 6; 0.5 mg/ml in well 5).
1b. Similar to 1a but center well was filled with 'absorbed' anti-procaine-BSA serum.
1c. Wells 1 and 3: 'absorbed' anti-procaine-BSA serum.
Well 2: anti-procaine rat Gf serum.
Well 4: procaine-BSA (1 mg/ml).
1d. Similar to 1c but slide was washed with saline containing procaine (see Methods).

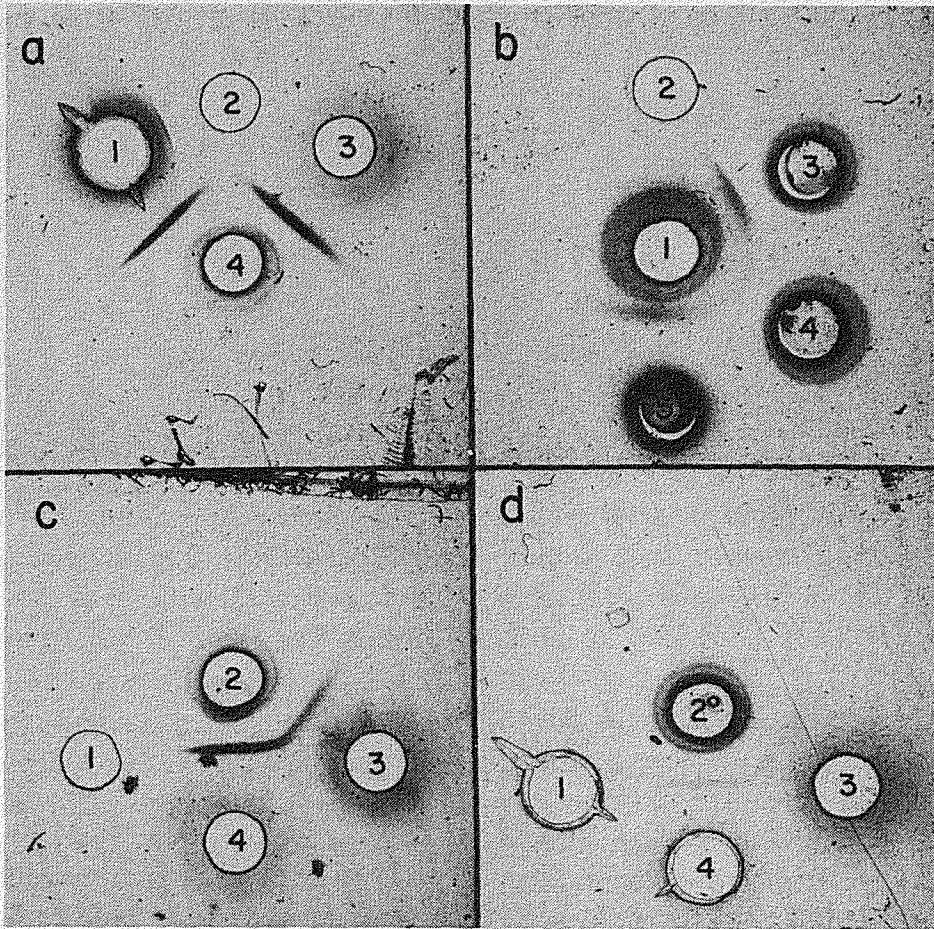


Fig. 2. Double immunodiffusion analysis of rabbit anti-procaine-protein sera.

- 2a. Well 1: anti-procaine-BSA serum.
Well 2: BSA (1 mg/ml).
Well 3: 'absorbed' anti-procaine-BSA serum.
Well 4: procaine-BSA (1 mg/ml)
- 2b. Well 1: anti-procaine rabbit Gf serum.
Well 2: BSA (1 mg/ml).
Well 3: procaine-BSA (1 mg/ml).
Well 4: procaine-rabbit Gf (1.2 mg/ml).
Well 5: procaine-rat Gf (0.9 mg/ml).
- 2c. Well 1: carbodiimide treated BSA (1 mg/ml).
Well 2: procaine-BSA (1 mg/ml).
Well 3: anti-procaine-rabbit Gf serum.
Well 4: 'absorbed' anti-procaine-BSA serum.
- 2d. Similar to 2c but slide was washed with saline containing procaine (see Methods).

The specificity of the antibodies produced was directed towards both the carrier molecule and the procaine group. Removal of the anti-BSA antibodies resulted in sera that reacted only with procaine-BSA. These sera were also tested in immunodiffusion experiments. Fig. 1a shows a rat anti-procaine-BSA serum, in the center well, reacting with procaine-BSA (well Nos. 1-3) and BSA (well Nos. 4-6). It is evident from this slide that the precipitin line formed with BSA fuses only partially with the precipitin line formed against the procaine-BSA conjugate. In Fig. 1b,

the anti-BSA antibodies have been removed from the antiserum, and there is only one precipitin line formed between the 'absorbed' serum and the procaine-BSA conjugate. Furthermore, as shown in Fig. 1c, procaine-BSA (well No. 4) reacts not only with the anti-procaine-BSA serum (well Nos. 1 and 3), from which the antibodies to BSA have been removed, but reacts also with rat anti-procaine-rat Gf serum (well No. 2). The bands fuse with each other in a pattern of identity. Fig. 1d is a duplicate of Fig. 1c but it was washed with saline containing pro-

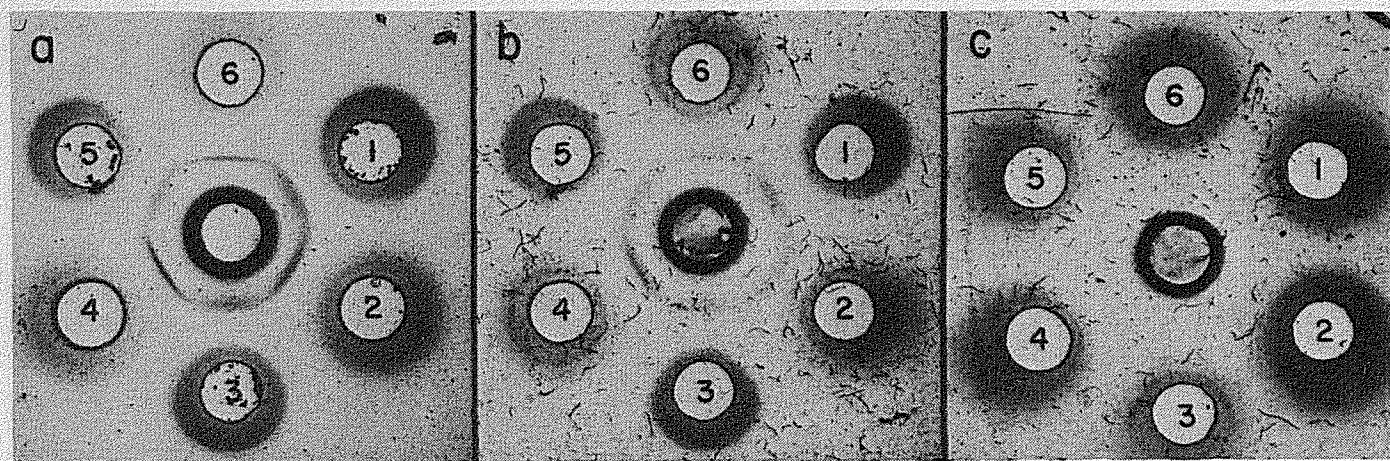


Fig. 3. Double immunodiffusion analysis of rat and rabbit anti-procaine-protein sera.

- 3a. Center well: procaine-BSA (1 mg/ml).
Wells 1, 3, 5: rat anti-procaine-rat Gf sera.
Wells 2, 4, 6: rabbit anti-procaine-BSA sera.
- 3b. Center well: procaine-BSA (1 mg/ml).
Wells 1, 3, 5: rat anti-procaine-BSA sera.
Wells 2, 4, 6: rabbit anti-procaine-rabbit Gf sera.
- 3c. Similar to 3a but slide was washed with saline containing procaine (see Methods).

caïne (see Methods) before drying and staining. The precipitin bands which had been formed between the sera and the antigen were completely dissolved indicating that the antibodies in both sera were specific for the procaine group. The anti-procaine-BSA sera from which antibodies to BSA were removed and the anti-procaine-rat Gf sera did not react at all with the carbodiimide-treated carrier. Procaine-rat Gf as antigen reacted with antisera to both procaine-protein conjugates but the precipitin bands formed were very faint as most of the antigen had precipitated around the antigen well.

Immunogenicity of procaine-protein conjugates in rabbits. Rabbits were immunized with either procaine-BSA (18 and 30 mol procaine/mol BSA) or procaine₂₉-rabbit Gf, the latter used mainly to avoid production of antibodies to the protein carrier. Sera from rabbits immunized with these antigens were tested in double diffusion experiments for the presence of precipitating antibodies to the procaine group. When procaine-BSA was the immunizing antigen the sera contained detectable amounts of antibodies to the protein carrier in addition to antibodies reacting with the procaine-protein conjugate (Fig. 2a).

Antibodies to the carrier molecule could be easily removed by absorption with BSA. Immunization with procaine-rabbit Gf did not induce antibody production to the protein carrier. Carbodiimide-treated BSA did not react with the anti-procaine-BSA serum from which antibodies to BSA were removed (Fig. 2c). Antisera to procaine-rabbit Gf gave precipitin bands when they were allowed to diffuse against either procaine-BSA, procaine-rabbit Gf or procaine-rat Gf (Fig. 2b). The latter two antigen solutions, for this plate, were prepared by dissolving 5 mg of the conjugate per ml saline and centrifuging the resultant solution to remove the non-soluble material. Similarly, antisera to either procaine-BSA or procaine-rabbit Gf reacted with procaine-BSA and the precipitin bands fused with each other in a pattern of identity (Fig. 2c). The precipitin bands between antigen and antibody were almost completely dissolved when the slides were washed in saline containing procaine at a concentration of 10 mg/ml (Fig. 2d). A faint band left after such washing suggested that antibodies with specificity towards the molecular configuration at the site of combination of hapten and carrier had been produced in these animals.

Cross-reaction of antisera of rat and rabbit origin. Antisera to procaine-protein conjugates (antibodies to carrier protein in the case of BSA were removed by absorption) obtained from rats or rabbits reacted with procaine-BSA as antigen in immunodiffusion experiments giving continuously fusing bands. Fig. 3a shows three different rat anti-procaine-rat Gf sera (well Nos. 1, 3 and 5) and rabbit anti-procaine-BSA sera (well Nos. 2, 4, and 6) reacting with procaine-BSA in the center well. Fig. 3b shows three different rat anti-procaine-BSA sera (well Nos. 1, 3 and 5) and rabbit anti-procaine-rabbit Gf sera (well Nos. 2, 4 and 6) reacting with procaine-BSA in the center well. All precipitin lines fused with each other in a pattern of identity. When the duplicate of slide 3a was washed with saline containing procaine at a concentration of 10 mg/ml the precipitin bands were almost completely dissolved leaving only very faint lines opposite the rabbit antisera (Fig. 3c). The precipitin bands, being extremely faint, cannot be seen in the picture, but were seen in the original slide. These results showed that regardless of the antigen and the animal species used for immunization antibodies were produced towards the common antigenic determinant, procaine.

DISCUSSION

Allergic reactions to drugs are quite common (3, 4, 13) and involve a number of drugs to various degrees. This points to the fact that a large number of drugs have the potential of inducing this type of reaction in man. Animal experimentation has not been generally useful in giving information concerning the sensitizing properties of drugs. One of the main difficulties is due to the fact that it is extremely difficult to mimic in the experimental animal the reaction seen in man. The discovery of the IgE immunoglobulin and its role in allergic reactions (5) as well as the implication of certain subclasses of IgG immunoglobulin (11, 16, 19) in allergy has helped in the elucidation of the mechanisms of the allergic reactions in man. Homocytotropic antibodies which are similar to human IgE have been detected in experimental animals after certain immunization procedures (2), and another type of

antibody with anaphylactic nature has been identified as a subclass of IgG in several animal species (2). Thus, it was worth investigating the possibility of finding an animal model which will give information concerning the sensitizing potential of drugs, not by trying to mimic in animals the allergic reaction as seen in man, but by measuring anaphylactic antibody response in animals on immunization with drug-protein conjugates.

The prevailing idea in drug allergy is that drugs after activation by liver microsomal enzymes react with proteins *in vivo* and are thus rendered immunogenic (18). Allergy to drugs is certainly dependent on protein reactivity of the drug, or rather its metabolites, and the structural characteristics of the drug. However, one of the factors influencing the incidence of drug allergy which is low in most cases, must be the formation and the amount of the immunogenic intermediate. By using a preformed drug-protein conjugate and not depending on its *in vivo* formation, which can be influenced by a number of factors, we would expect a higher antibody response and a higher incidence for this response and thus a more sensitive test. Considering that the antibody response to procaine determinants is not very high on immunization with procaine-protein conjugates it is doubtful if procaine alone would induce, under these experimental conditions, a significant and measurable antibody response. This is the reason why we have not exposed rats to the free drug but we have used instead drug-protein conjugates.

Procaine-protein conjugates were prepared (Table 1) using the carbodiimide method, and their immunogenicity was studied in the rat. It was found that these conjugates were immunogenic with respect to IgG antibody production (Table 2) but IgE type of antibodies could not be easily detected towards the immunogen. Precipitating antibodies were also detected in rat sera. The class these antibodies belong to was not characterized. The specificity of antibodies produced was directed towards the procaine group and when BSA was used as carrier towards the carrier protein (Table 2, Fig. 1). Antisera to the procaine group could be obtained

after absorption with BSA (Table 2, Fig. 1). Rat Gf consisting of homologous proteins did not elicit any antibody response in rats. Antibodies to carbodiimide-treated carriers were not detected, suggesting that under the experimental conditions employed no undesirable transmolecularly cross-linked protein or dicyclohexylurea-protein derivatives (6) were formed to appreciable degree. Hapten inhibition studies revealed that the antibodies in the sera of animals immunized with procaine-rat Gf and procaine-BSA (after absorption with BSA) were specific for the procaine group. Procaine-rat Gf did react with the anti-procaine sera in immunodiffusion experiments but the bands were very faint as most of the antigen precipitated in and around the antigen well.

The immunogenicity of the procaine-protein conjugates was further studied in rabbits with respect to precipitating antibodies. The results in rats were to a considerable degree reproduced in rabbits as far as specificity of antibodies was concerned. Antibodies were detected towards the carrier protein (BSA), the procaine molecule and the molecular configuration of the site of combination of the hapten and carrier (Fig. 2). Specificity of antibodies was established by absorption experiments (Fig. 2a) and by hapten inhibition experiments (Fig. 2d). Antibodies produced against procaine-BSA or procaine-rabbit Gf showed similar specificity with regard to anti-hapten antibodies and gave a pattern of identity in immunodiffusion studies (Fig. 2b, 2c). As with rat, rabbit sera did not react with the carbodiimide-treated carrier. Procaine-rabbit Gf as antigen gave similar problems as the procaine-rat Gf with respect to its solubility.

In our previous studies with penicillin (8, 9, 17) the production of both IgE and IgG_a antibodies to the penicilloyl group was observed. The inability of procaine-protein conjugates to induce IgE type antibodies to the procaine group may be related to the lower degree of allergenicity of procaine in man when compared with penicillin. The fact that IgG_a antibodies were produced to the procaine group still suggests that procaine has the potential to participate in anaphylactic reactions.

The feasibility of using the antibodies raised

in rabbits against the procaine group for the development of a radioimmunoassay test for determining procaine is under investigation.

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