

Isolation of Allergenicity Active Cytochrome c from Kentucky Blue Grass Pollen¹

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Abstract. An allergen, designated as Kentucky blue grass KBG-cytochrome c, was isolated from the nondialysable constituents (R) of the aqueous extract of KBG pollen by a combination of gel filtration and preparative isoelectrofocussing techniques. The allergen had an absorption spectrum characteristic of heme protein and had a molecular weight corresponding to 12,000 daltons. It was a basic protein with a pI value of 9.9 and contained all the common amino acids. KBG-cytochrome c elicited immediate hypersensitivity cutaneous reactions in individuals allergic to KBG pollen.

Introduction

Cytochrome c has been extensively used as a prime model for establishing the evolutionary developments leading to the divergence of eukaryotes. The phylogenetic relationships of these organisms have been obtained from the comparative analysis of the amino acid sequence data of cytochromes c. As a result of this interest in the evolutionary history, the primary structures of 85 cytochromes c of different origin have been elucidated [4]. Although cytochromes c of diverse origin, e.g. horse, bonito and tuna, possess a wide variation in amino acid se-

quence, they all have an identical 3-dimensional structure. Thus, cytochromes c represent an excellent group of proteins for studying the effects of differences in amino acid sequence on the antigenicity of a globular protein with unvarying conformation. Several investigators [3, 16, 23-27] have used cytochrome c as a model antigen for localizing antigenic determinants within the molecule and also for dissecting the cellular immune response to a globular protein. These studies were accomplished by relating the extent of the immune cross-reactivity at both the humoral and cellular levels, of various cytochromes c to differences in their amino acid sequences.

The recent finding of Goodfriend et al. [14] that cytochrome c isolated from ragweed pollen was allergenic in ragweed-sensitive individuals suggests the possibility

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that cytochromes c present in other pollens may also have allergenic activity. If other pollen cytochromes c are indeed found to be allergenic, it would be of interest to determine the extent, if indeed any, of their allergenic cross-reactivity.

Because the structural features of different cytochromes c have been highly conserved during evolutionary changes, pollen cytochromes from related species within a family, e.g. Graminae (grass), may be expected to differ minimally in their amino acid sequences. These minor differences may help to identify specific regions of the cytochrome molecule which are responsible for the allergenic activities. This approach would be similar to that which has been successfully used to identify three regions within the primary structure that have been implicated in the antigenicity of mammalian cytochromes c (e.g. human, horse, guanaco and mouse) [23].

In earlier studies from our laboratory, protocols were developed for isolating purified allergens from the aqueous extract of KBG pollen [1, 2, 10–12]. In the present study, we report the isolation of an allergenically active cytochrome c from the nondialyzable multicomponent fraction, i.e. retentate (R), of the aqueous extract of Kentucky blue grass (KBG) pollen by a combination of gel filtration and isoelectrofocussing techniques.

Materials and Methods

Preparation of R

An aqueous extract of KBG pollen (Hollister-Stier Laboratory, Mississauga, Ont., Canada) was prepared according to the procedure previously established in this laboratory [7]. The aqueous extract was dialyzed through visking tubing, No. 20 dialysis (Union Carbide Canada Ltd., Lindsay,

Cnt., Canada) against distilled water for 48 h at 4 °C; the nondialyzable components are referred to as the retentate (R).

Chromatography

Sephadex G-50 (particle size 20–80 µm; Pharmacia AB, Uppsala, Sweden) was equilibrated with 0.05 M Tris-HCl, pH 8.6, containing 0.02% NaNa₃ and packed into a column (2.5 × 90 cm) at 4 °C.

Ultrogel ACA-44 (LKB 2204–440, Industrie Biologique Française, Genevilliers, France) was equilibrated with 0.05 M phosphate buffer, pH 7.2, containing 0.15 M Na₂SO₄, 0.02% NaNa₃ and packed into a column (2.5 × 90 cm).

All chromatographic eluates were concentrated by ultrafiltration through UM-2 membrane, with a cutoff for molecules larger than 1,000 daltons (Amicon, Lexington, Mass.). Desalting of the concentrated eluates was achieved by dialysis through Spectrapor membrane tubing (specified molecular weight cutoff = 3,500; Spectrum Medical Industries, Inc., Los Angeles, Calif.) for 24 h against a large volume of distilled water with three changes.

Preparative Isoelectrofocussing (ISO-EF)

The procedure of ISO-EF recently described [11] was adapted with the following modifications. Thus, 5.5 g of Ultrodex (LKB-Product AB, Stockholm-Bromal, Sweden) was swollen in 142.5 ml of a solution of fraction R-1 (see Results, p. 370) at a concentration of 4 mg/ml water. A volume of 7.5 ml of 40% ampholyte buffer, pH range 3.5–10 (LKB), was added to the gel suspension, which was then deaerated under vacuum. The gel was poured onto a horizontal glass tray (11 × 30 × 0.8 cm, Bio-Rad Laboratories, Richmond, Calif.), spread uniformly and dried to a weight loss of about 30%. Three strips of paper wicks (0.7 × 11 cm, Bio-Rad) presoaked with 0.2 M H₂SO₄ for the anode and another three strips presoaked with 0.4 M ethylenediamine for the cathode were then laid on top of the gel at the far ends; ISO-EF electrodes, 110 mm (Bio-Rad) were placed on top of the wicks and the ISO-EF was carried out as described previously [11].

After ISO-EF, the gel was divided into 38 equal sections with the aid of a gel divider (Bio-Rad) and the pH of each gel section was measured with the aid of surface pH electrode (Bio-Rad). Each gel section was poured into a PEGG elution column (LKB) and eluted with 5 ml of water. The

absorbance of the eluates at 280 nm was measured in a Perkin-Elmer UV-VIS spectrophotometer (Coleman 139, Coleman Instrument Division, Maywood, Ill.). All fractions were dialyzed extensively against water through Spectrapor membrane tubing (molecular cutoff = 6,000–8,000) and lyophilized.

Protein Measurement

The protein content of pollen fractions was determined by the method of Lowry et al. [21]. Unless stated otherwise, amounts or concentrations of various fractions are expressed in terms of their protein content.

Absorption Spectrum

The sample was dissolved in water and read at wavelengths of 5-nm intervals in a Zeiss spectrophotometer (Zeiss, Oberkochen, FRG).

Chemical Analysis

The amino acid analysis was kindly performed by Dr. F. Stevens of the Department of Biochemistry, University of Manitoba. Samples were hydrolyzed with HCl at 110–112 °C for 24, 48 and 72 h. The hydrolysates were analyzed with the Beckman automatic amino acid analyzer, Model 120–139 (Beckman, Palo Alto, Calif.). The concentration of serine, threonine and tyrosine was calculated by extrapolation to zero time hydrolysis and that of valine and isoleucine was calculated from the analysis of 72 h hydrolysate. The cysteine was determined as cysteic acid by oxidizing the sample with performic acid [15] prior to 24 h acid hydrolysis. The tryptophan content was measured spectrophotometrically [6]. The concentration of other amino acids was calculated from the analysis of the 48 h hydrolysate. Qualitative tests for carbohydrate were performed by a spot test as described elsewhere [11].

Molecular Weight Estimation

The molecular weight of pollen constituents was determined by electrophoresis in polyacrylamide gel in presence of sodium dodecyl sulfate (SDS-PAGE) as described by Laemmli [18]. The separation gel consisted of 10 g acrylamide, 0.8 g methyl bis-acrylamide, 1 g SDS, 0.4 g ammonium persulfate dissolved in total volume of 100 ml of 0.375 M Tris-HCl, pH 8.8. The gel was polymerized by adding 0.025 ml of tetramethylethylenediamine (TEMED) to the 10 ml of gel mixture and poured into glass tubes (0.5 × 12.5 cm). The stacking gel was prepared in the same way, except that the concentration of acrylamide and methyl bis-acrylamide was 3 and 0.8%, respectively, in 0.125 M Tris-HCl, pH 6.8. The electrode buffer (pH 8.3) contained 0.025 M Tris and 0.192 M glycine and 0.1% SDS. The sample buffer consisted of 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue. The sample was dissolved in sample buffer and boiled for 90 s. Electrophoresis was carried out with a current of 3 mA/tube until the bromophenol blue marker reached the bottom of the gel. The gels were stained according to the method of Fairbanks et al. [13]. The following proteins with the indicated molecular weights were used as markers: human serum albumin, 68,000 (Pentex, Miles Laboratories, Elkhart, Ind.); ovalbumin, 43,000 (ICN Pharmaceuticals, Cleveland, Ohio); chymotrypsinogen A, 25,700, ribonuclease A, 13,700 (Worthington Biochemical Corp., Freehold, N.J.); pepsin, 35,000 trypsin, 23,300, horse heart cytochrome c, 11,700 (Sigma, St. Louis, Md.). The mobility of the separated protein was measured as described by Weber and Osborn [28].

Alternatively, the molecular weights of pollen fractions were estimated by gel filtration through Ultrogel ACA-44 calibrated with proteins listed above.

Radioallergosorbent Test (RAST)

The test recently described [11] was used with the following modifications. CNBr-activated paper discs (one disc/100 µg of sample to be coupled) were suspended in a solution of a pollen fraction (1.0 mg/ml of 0.1 M NaHCO₃) for 18 h at 4 °C. Unreacted materials were removed by washing the paper discs twice with 0.1 M NaHCO₃. To block any residual active sites on the cellulose, the discs were reacted with a solution of β-ethanolamine (0.5 M in 0.1 M NaHCO₃) for 3 h and then washed with 0.1 M sodium acetate buffer, pH 4.0, followed by extensive washing with phosphate-buffered saline (7.75 g NaCl, 0.54 g KH₂PO₄, 2.14 g Na₂HPO₄ in liter of H₂O, pH 7.2; PBS). The discs were stored in incubation buffer (PBS containing 0.3% bovine serum albumin and 0.02% NaNa₃).

Volumes of 0.05 ml of a pool of human sera from 6 patients allergic to KBG were incubated overnight at 4°C with the allergosorbent discs and then washed with incubation buffer. The extent to which the IgE antibodies bound to the insolubilized allergens was evaluated by the addition of ¹²⁵I-labelled rabbit anti-human IgE antibodies bound to the discs was measured with a Beckman Gamma 300 System counter.

For the experiments involving the inhibition of RAST, 0.05 ml volume of allergic sera was mixed with different amounts of each pollen fraction and maintained for 3 h at room temperature and then added to the allergosorbent discs.

Skin Tests

The skin tests were kindly performed by Dr. R. Warrington of this Department. A stock solution of 0.1 mg/ml of fraction in saline was sterilized by passing through a sterile Millipore membrane (0.45 µm pore size). A 20-µl volume of a 100-fold diluted stock solution was injected intradermally into patients sensitive to KBG. 15 min later, the size of the wheal was measured.

Passive Cutaneous Anaphylaxis (PCA)

This procedure was employed to establish the PCA activity of isolated fractions relative to that of R. For this purpose, IgE antibodies to the allergens present in R were produced in mice and were determined in terms of their PCA titer on the skin of rats, as previously described [8, 9]. For the actual determination of PCA activity, hooded rats were locally sensitized by the intradermal injection of a volume of 50 µl of the mixture and 24 h later the PCA reaction, if any, was elicited by intravenous (i.v.) administration of 0.1 mg of a pollen fraction in Evan's blue dye (1% in saline); a PCA reaction was considered positive when the extent of the extravasation of the dye, as measured on the underside of the skin, was 5 mm in diameter or greater.

Results

The retentate fraction, R, was resolved by chromatography on Sephadex G-50 into two fractions arbitrarily designated as R-I

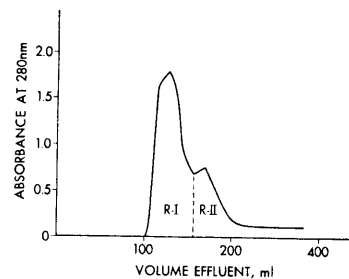


Fig. 1. Gel filtration of R on Sephadex G-50 (particle size, 20–80 µm). Column size 2.5 × 90 cm; eluting buffer: 0.05 M Tris-HCl, pH 8.6, containing 0.02% NaN₃.

and R-II (fig. 1). The chromatography also resulted in the removal of some low molecular weight yellow-pigmented materials, which were retarded in the column and, therefore, were eluted much later than R-II. It was found that the absorption of fraction R-I at 410 nm relative to its absorption at 280 nm was higher than that of R, whereas R-II contained very little material which absorbed at 410 nm. Since the absorption at 410 nm is one of the characteristic absorption maxima of cytochrome c, it would appear that the gel filtration of R yielded a fraction, i.e. R-I, which was enriched in cytochrome c content. Fraction R-I was therefore rechromatographed on Sephadex G-50 and then subjected to preparative ISO-EF on Ultrodex in the pH range of 3.5–10. The gel containing the focussed components was subdivided into 38 fractions, including anode and cathode fractions, i.e. the fractions underneath the electrode wicks. As is shown in figure 2, fraction R-I was resolved into several components. A distinct red-colored band was localized within the cathode fraction.

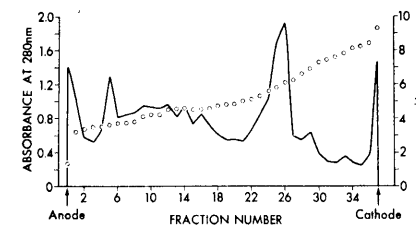


Fig. 2. Preparative ISO-EF of R-I on Ultrodex (ampholine: pH 3.5–10; 96 h).

Table I. Allergic activity of focussed fractions of R-I

Fraction No.	Inhibition of RAST ¹ , %	Fraction No.	Inhibition of RAST ¹ , %
Anode (A)	70.0	19	59.0
1	68.5	20	65.4
2	42.9	21	65.0
3	51.8	22	71.5
4	30.5	23	73.8
5	33.0	24	72.3
6	34.0	25	64.0
7	40.5	26	77.4
8	33.7	27	72.8
9	29.5	28	72.4
10	23.4	29	74.7
11	25.3	30	64.7
12	34.0	31	58.0
13	42.6	32	59.7
14	46.5	33	57.0
15	49.8	34	55.0
16	55.0	35	57.6
17	57.9	36	52.6
18	59.3	cathode (C)	51.9
		R	90.7

¹ 100 µg (protein) of each fraction or of R was incubated with a pool of sera from humans allergic to KBG pollen, prior to addition to the allergosorbent disc prepared with R.

Allergic Activity of Subfractions of R-I

The allergic activity of these 38 subfractions was evaluated by inhibition of the RAST utilizing allergosorbent discs prepared with R and a pool of human anti-KBG reaginic sera. The results listed in table I indicate that all 38 subfractions inhibited the RAST to varying degrees and, therefore, indicated that they all possessed allergenically active components.

Isolation of Cytochrome c from Cathode Fraction

An analysis of the absorption spectrum of the cathode fraction showed that it had absorption maxima at 280, 410, 520 and 550 nm and similar to that of cytochrome c. SDS-PAGE analysis revealed that the cathode fraction contained three components with molecular weights corresponding to 53,000, 28,500 and 12,000 daltons. These components were further resolved by chromatography on Ultrogel ACA 44 (fig. 3). Thus, five fractions, designated as I, II, III,

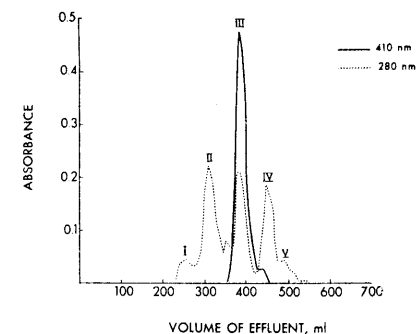


Fig. 3. Gel filtration of cathode fraction on Ultrogel ACA 44. Column size 2.5 × 90 cm; eluting buffer: 0.05 M phosphate buffer, pH 7.2, containing 0.15 M Na₂SO₄ and 0.02% NaN₃.

IV and V contained material with high optical density profiles at 280 nm. However, only fraction III showed high absorption at 410 nm, while fraction IV contained a small amount of material which absorbed at 410 nm. Fraction III was rechromatographed on a column of Ultrogel ACA 44 calibrated with proteins of known molecular weight and it was found that the elution volume of fraction III corresponded to that of horse cytochrome c. Fraction III will hereafter be referred to as KBG-cytochrome c.

Immunological and Physicochemical Properties of KBG-Cytochrome c

It was shown by the RAST procedure that the allergosorbent disc prepared with 100 μ g of KBG-cytochrome c and incubated with 50 μ l of a pool of the allergic sera was capable of binding 20% of ^{125} I-antihuman IgE added, as compared to 37% of the radioactivity bound by the allergosorbent disc prepared with 100 μ g of R. Further evidence for the allergenicity of KBG-cytochrome c was obtained by direct skin testing of 3 KBG-sensitive patients (whose sera were not included in the pool of sera used in RAST). KBG-cytochrome c at a concentration of 1 μ g/ml gave positive skin reactions in 2 patients, the size of the wheal being 12 $\times$ 8 and 8 $\times$ 8 mm, respectively. The 3rd patient did not react to this challenge. Consistent with this finding, KBG-cytochrome c was also demonstrated to be capable of eliciting PCA reactions in rats passively sensitized with a murine anti-R reagenic sera. The PCA titer of the murine reagenic sera obtained by i.v. challenge of 100 μ g of KBG-cytochrome c was 160 (PCA titer elicited with 100 μ g of R was 640).

The absorption spectrum of KBG-cytochrome c is shown in figure 4. Thus,

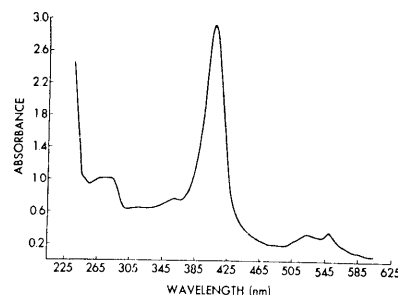


Fig. 4. Absorption spectrum of KBG-cytochrome c (0.7 mg/ml H₂O) at room temperature.

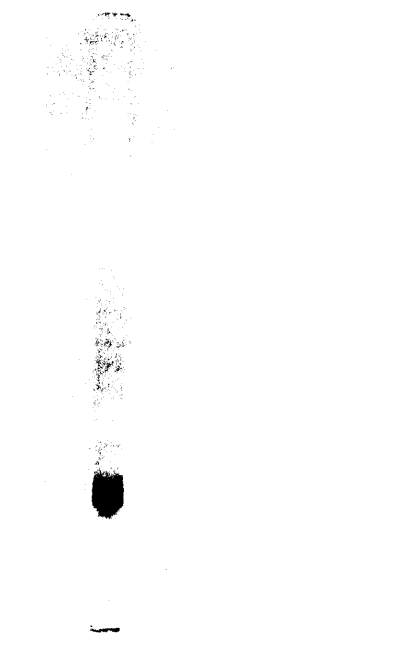


Fig. 5. Polyacrylamide gel electrophoresis of 50 μ g protein of KBG-cytochrome c in presence of SDS.

Table II. Amino acid composition of KBG-cytochrome c

Amino acid	Residue per molecule
Alanine	9
Arginine	4
Aspartic acid	10
Cysteine	2
Glutamic acid	11
Glycine	10
Histidine	2
Ileucine	3
Leucine	8
Lysine	11
Methionine	2
Phenylalanine	5
Proline	9
Serine	5
Threonine	7
Tryptophan	2
Tyrosine	4
Valine	8

KBG-cytochrome c had absorption maxima at 280, 350, 410, 520 and 550 nm. While the absorption maxima at 280 nm is due to protein, the maxima at 350, 410, 520 and 550 are the characteristics of the hemochrome spectrum of cytochrome c [22]. KBG-cytochrome c is red in color; if, however, a few crystals of sodium dithionite were added to a solution of KBG-cytochrome c, the color changed to pink. Under this reduced conditions, the absorption maxima at 410 shifted to 415, the absorption maxima at 550 was more pronounced while the absorption maxima at 520 was barely detectable. These data would indicate that cytochrome c, isolated from KBG pollen, was predominantly in oxidized form.

KBG-cytochrome c was shown to be homogeneous, since it moved as a single

band corresponding to a molecular weight of the order of 12,000 daltons in SDS-PAGE (fig. 5). The pI of KBG-cytochrome c, as determined by preparative ISO-EF of KBG-cytochrome c was 9.9. The amino acid analysis revealed that it contained all the naturally occurring amino acids (table II). KBG-cytochrome c did not contain carbohydrate, since it failed to give a positive test for carbohydrate.

Discussion

In this study allergenically active cytochrome c was isolated from the nondialysable fraction R of KBG pollen. The constituents of R were first separated, on the basis of their size, into two fractions and the higher molecular weight fraction, R-I, which was enriched with cytochrome c like material, was further resolved by preparative ISO-EF into fractions with pI values ranging from 3.5 to 10.

Although all the focussed fractions of R-I contained allergenic components, only the cathode fraction was enriched with red-colored material and had an absorption spectrum characteristic of cytochrome c. SDS-PAGE analysis revealed that the cathode fraction contained a component with a molecular weight characteristic of cytochrome c and two additional components with molecular weights higher than that of cytochrome c. The cathode fraction was resolved on ACA 44 into five fractions, all of which were capable of binding human IgE antibodies to allergens of KBG pollen. The two low molecular weight fractions, i.e. fractions IV and V, were easily dialysable through Spectrapor membrane tubing (molecular cut off = 6–8 $\times$ 10³) and, conse-

quently, were not detectable by SDS-PAGE analysis.

Fraction III (fig. 3) was identified as KBG-cytochrome c by its color, absorption spectrum, size, charge and amino acid composition similar to those cytochromes isolated from other plant sources [4]. Although KBG-cytochrome c is a highly basic protein (pI value = 9.9), the acidic amino acids, i.e. aspartic acid and glutamic acid, were found to be present in amounts higher than the basic amino acids, i.e. arginine, histidine and lysine. Since asparagine and glutamine in a protein are converted into glutamic and aspartic acids, respectively, during acid hydrolysis, the amounts of aspartic and glutamic acid determined by the acid hydrolysis of KBG-cytochrome c may indeed be higher than their actual values. To obtain the true values, it will be necessary to identify the individual amino acids in the course of the complete sequence analysis of KBG-cytochrome c. A striking difference noted between KBG-cytochrome c and two glycoprotein allergens, C-I-2-6 [11] and allergen C [2] also isolated from KBG pollen, was that the cytochrome c allergen was devoid of carbohydrate. An analysis of allergenic relationships between the two glycoprotein allergens and KBG-cytochrome c may aid in the understanding of the role of carbohydrate (if any) on the allergenicity of the two glycoprotein allergens. It should be noted that the color, size and charge characteristics of cytochrome c closely resembled that of allergen C [2] and one may therefore contend that cytochrome c might be contaminated with allergen C. Although such a possibility cannot be completely ruled out, the following observations would suggest that allergen C, if at all present in KBG-cytochrome c, it would be in trace amounts.

Thus, KBG-cytochrome c did not precipitate with rabbit antiserum prepared against allergen C. Allergen C contained approximately 30% carbohydrate whereas no carbohydrate was detected in KBG-cytochrome c. A complete analysis of antigenic and allergenic relationships of KBG-cytochrome c and allergen C must await the availability of appropriate antisera to KBG-cytochrome c.

The salient feature of cytochrome c isolated from KBG pollen was that it elicited allergic reactions in individuals sensitive to KBG pollen. The fact that cytochrome c of KBG and ragweed pollen were demonstrated to be allergenic in man strongly suggests that the various pollens may contain cytochrome c as a part of their allergenic constituents. As referred to in the Introduction, the isolation of cytochromes c of various grass pollens may represent a group of cross-reactive allergens. The availability of well-characterized and closely-related allergens is expected to pave the way for localizing the allergenic sites on the molecule by correlation of allergenic activity with distinct region(s) identified from amino acid sequence analysis. Once the allergenic determinant(s) are identified, it can be anticipated that the synthesis of univalent derivatives of the allergenic determinants may be achieved.

The univalent determinants would serve as immunotherapeutic agents, since these compounds on administration would inhibit the allergic reactions triggered by the cross-linking of cell-fixed IgE antibodies by multivalent allergens. For example, the treatment of penicillin allergy by the injection of large doses of the univalent penicillin derivative, benzylpenicilloyl-formyl lysine, prior to administration of penicillin to sensitive patients, was successfully achieved by *de*

Weck and Girard [5]. Alternatively, the allergenic determinants could be incorporated into tolerogenic conjugates for abrogating the IgE antibody response, as has been achieved in experimental animals by the injection of conjugates of the appropriate hapten (e.g. benzylpenicilloyl or 2,4-dinitrophenyl groups) with nonimmunogenic carriers, such as isologous γ -globulins [19], polyvinyl alcohol [20] or the synthetic copolymer of *D*-glutamic acid and *D*-lysine [17].

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