Journal of Chromatography, 597 (1992) 365–376
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 953

Separation of monoclonal antibodies from antihapten antisera by two-dimensional affinity electrophoresis

Kazusuke Takeo*, Kazuyuki Nakamura and Ryosuke Suzuno

The First Department of Biochemistry, Yamaguchi University School of Medicine, Kogushi-1144, Ube 755 (Japan)

ABSTRACT

A high-resolution two-dimensional affinity electrophoresis (2D-AEP) method was developed, using capillary polyacrylamide gel (PAG) isoelectric focusing in the first and slab PAG affinity electrophoresis in the second direction. Using this method, anti-hapten antibodies were separated into a number of monoclonal antibody [immunoglobulin G (IgG)] families, each of which is composed of several IgG spots having an identical affinity to the hapten but different isoelectric points. 2D-AEP may offer a powerful tool for solving fundamental problems in immunochemistry such as antibody heterogeneity, its hapten binding specificity and antigen-dependent somatic mutation.

INTRODUCTION

Induced antibodies against a single antigenic determinant are highly heterogeneous. The resolution of such heterogeneous antibodies into each individual immunoglobulin is essential in order to determine the molecular diversity of the antibodies and their binding characteristics. For this purpose, isoelectric focusing (IEF) or two-dimensional electrophoresis has generally been used. In spite of their high resolving power, satisfactory resolution still has not been attained. Two-dimensional electrophoresis, based first on the diversity of electric charge and second on differences in biological binding activity, should be efficient for separating heterogeneous protein mixtures of equal molecular mass, such as immunoglobulin. On this basis, we have developed a new type of two-dimensional electrophoresis [1,2], utilizing IEF in the first and affinity electrophoresis (AEP) [3] in the second direction; we call it two-dimensional affinity electrophoresis (2D-AEP). In this paper, we present the experimental 2D-AEP procedure for rabbit anti-hapten antibodies. Its resolving power for anti-hapten antibodies is discussed. The cross-reactivity of anti-hapten antibodies to various haptens can be examined by this method.

EXPERIMENTAL

Materials

Hapten-conjugated lysine Sepharoses and affinity ligands, hapten–carrier protein conjugates and anti-hapten antibodies were used.

All chemicals were of the highest available purity and were obtained from various sources.

Preparation of hapten-conjugated lysine Sepharoses

ε-N-Dinitrophenyllysine-Sepharose 4B (DNP-Sepharose). Add a tenfold molar excess of dinitrobenzene sulphonate (DNBS) in K_2CO_3 solution to a washed lysine-Sepharose 4B suspension with stirring at room temperature overnight in the dark using a rotary evaporator without suction. Wash the Sepharose beads extensively on a glass filter funnel. Store them in phosphate-buffered saline (PBS) (pH 7.4) containing 0.02% sodium azide at 4°C.

ε-N-Dansyllysine-Sepharose 4B (dansyl-Sepharose). Add a double molar excess of dansyl choride

0021-9673/92/$05.00

in acetone to the washed lysine-Sepharose suspension in 1% $NaHCO_3$ with gentle stirring overnight in a rotary evaporator without suction. Wash the Sepharose beads with acetone–PBS (1:1) and with PBS thoroughly. Store them as a suspension in PBS containing 0.02% sodium azide at 4°C, protected from light.

Preparation of macromolecular affinity ligands: hapten-conjugated non-cross-linked acrylamide–allylamine copolymer

Non-cross-linked acrylamide–allylamine copolymer. Dissolve 40 g of acrylamide and 4 g of allylamine in 400 ml of water in a 500-ml erlenmeyer flask, followed by addition of 0.8 ml of N,N,N′,N′-tetramethylethylenediamine (TEMED) and 50 ml of 0.8% freshly prepared ammonium peroxodisulphate solution with gentle mixing. Overlay water on the top of the solution carefully and allow the mixture to stand overnight in a water-bath at 30°C. Dialyse the reaction mixture extensively against tap water for 3 days, followed by deionized water with four changes per day for 2 days.

Dinitrophenylation of non-cross-linked acrylamide–allylamine copolymer (DNP-PA). Add 4.5 g of $NaHCO_3$ to the dialysed solution of the acrylamide–allylamine copolymer, followed by dropwise addition of 4.8 g of dinitrofluorobenzene in 15 ml of acetone. Stir the mixture overnight in the dark at 25°C using a rotary evaporator without suction. Dialyse the reaction mixture against tap water for 5 days and thereafter against deionized water with four changes per day for 2 days. Concentrate the dialysed DNP-PA solution *in vacuo* until the DNP concentration reaches 5–10 m*M* and store at 0–4°C in the dark. The concentration of the DNP residue was calculated from the absorbance at 360 nm in 0.1 *M* NaOH, using DNP-glycine as a standard [4] (molar absorptivity at 360 nm = 17 530 l mol^{-1} cm^{-1}).

Trinitrophenyl-conjugated acrylamide–allylamine copolymer (TNP-PA) can be prepared with a similar procedure using trinitrobenzene sulphonate in alkaline K_2CO_3 solution.

Dansylation of non-cross-linked acrylamide–allylamine copolyer (dansyl-PA). Add an amount of dansyl chloride in acetone equivalent to the amount of the amino groups of the allylamine residue of the copolymer in 1% $NaHCO_3$ with gentle stirring overnight at room temperature. Precipitate and solubilize the conjugate in 80% acetone and water repeatedly, and dialyse it against deionized water extensively. The concentration of dansyl residue of dansyl-PA was determined spectrophotometrically by assuming the molar absorptivity of dansyl residues at 330 nm at pH 7.5 to be 4570 l mol^{-1} cm^{-1} [5].

Preparation of hapten–carrier protein conjugates

Dinitrophenylated carrier proteins were prepared by coupling DNBS to a corresponding carrier protein in alkaline K_2CO_3 medium. Equivalent amounts of DNBS to lysine and tyrosine residues of the protein were added according to the method of Eisen [6]. The molecular ratio of the conjugated DNP residues to the carrier protein amounted to 25 per molecule for DNP-chicken serum albumin (CSA) and 99 per molecule for DNP-human γ-globulin (HGG). Dansyl-CSA was prepared by adding a double molar excess of dansyl chloride in acetone to lysine residue of CSA in 1% $NaHCO_3$ with gentle stirring overnight at room temperature in the dark. The reaction suspension was centrifuged and the supernatant was extensively dialysed against PBS. The molar ratio of the conjugated dansyl residues to the carrier protein was 99.

Preparation of anti-hapten antibodies

Anti-DNP antibodies. Rabbits were immunized with 5 mg of DNP-HGG or DNP-CSA in an emulsion with an equal volume of CFA (complete Freund adjuvant), injected intracutaneously into the dorsum every 4 weeks. A few millilitres of blood were taken from the animals periodically after each injection, and the antiserum obtained was directly applied to a DNP-Sepharose column (1 ml in a small column, 30 mm × 6.5 mm I.D.) which was equilibrated with PBS, and the column was washed thoroughly with PBS until the absorbance of the eluate at 750 nm by Lowry's protein assay became lower than 0.002. It was necessary to wash the columns at a flow-rate of 12 ml h^{-1} for 25–30 h. Anti-DNP antibody was then eluted from the column with 50 m*M* DNP-glycine (pH 7.4). Twenty drops (nearly 1 ml) of eluate were collected in each tube.

Volumes of 3 μl of eluate from each tube were spotted on a sheet of a nitrocellulose membrane and stained with 1% Ponceau Red 3R in ethanol–acetic

acid–water (20:10:70, v/v/v). The fractions containing protein were pooled (3–4 tubes) and dialysed against 500 ml of PBS with a change of PBS every 6 h until the sample solution became colourless. If the protein concentration of the dialysates was lower than 260 $\mu g\ ml^{-1}$, the dialysate was concentrated with a vacuum centrifuging concentrator. The purified samples were stored in the solution of 0.02% sodium azide in the dark below 0°C. The samples of anti-DNP antibody obtained consisted almost exclusively of the immunoglobulin G (IgG) class of immunoglobulin as tested by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), Ouchterlony double diffusion and immunoelectrophoresis. Some samples contained a minor fraction (less than 1%) of the IgM class of anti-DNP immunoglobulin, which was detected as a spot at the corner of the 2D-AEP patterns, corresponding to the position where the samples were applied. Non-specific IgG and other serum proteins were not detected.

Rabbit anti-dansyl antibodies. These were prepared by a similar procedure to that for anti-DNP antibodies. Briefly, rabbits were immunized in general with 5 mg of dansyl-CSA in an emulsion with an equal volume of CFA. Anti-dansyl antibodies were separated and purified from the antisera by loading on dansyl-Sepharose columns, followed by extensive washing with PBS and elution with dansyl-glycine at pH 7.4.

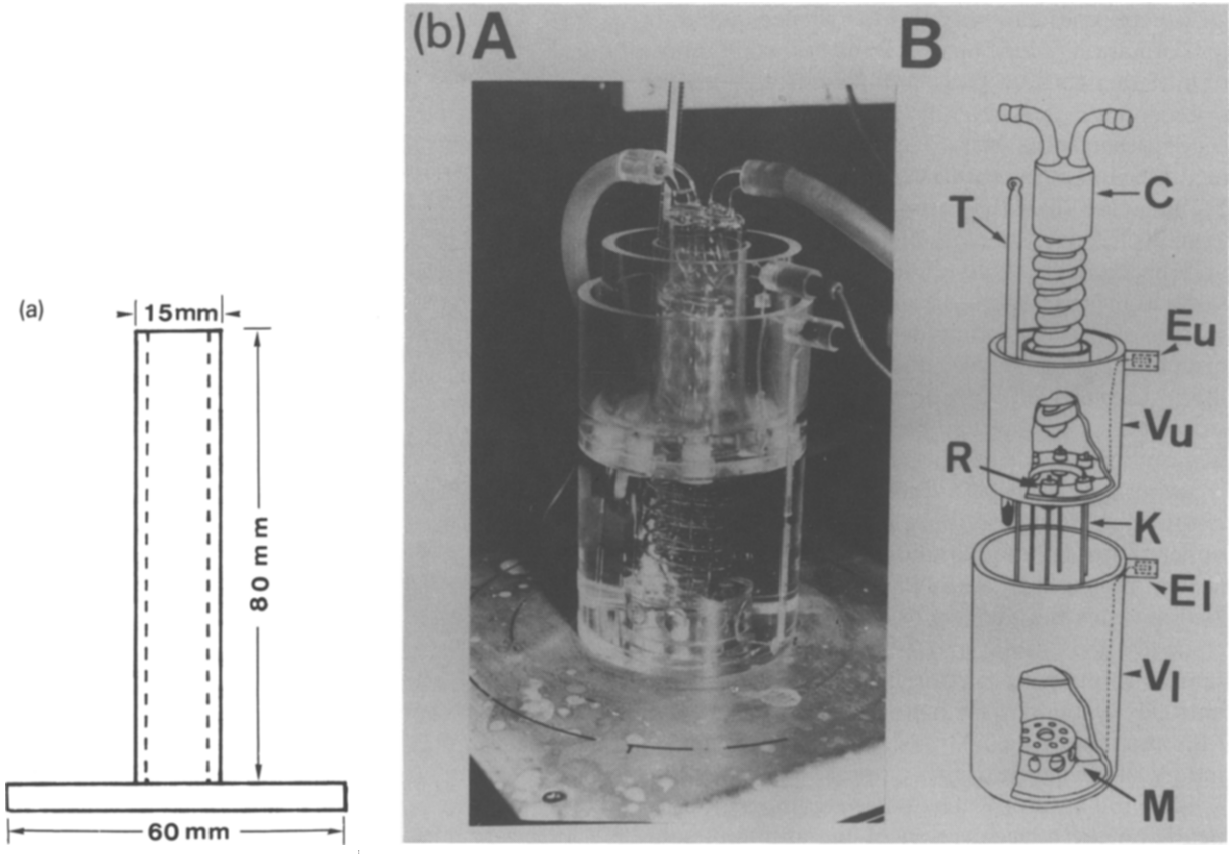


Fig. 1. (a) Diagram of plastic cylinder for preparation of IEF capillary tubes. (b) (A) Photograph and (B) diagram of thermostated capillary IEF apparatus. C = Glass condenser; E_l and E_u = lower and upper electrodes; K = IEF capillary tubes; M = magnetic stirrer; T = thermometer; V_l and V_u = lower and upper electrode vessels.

Two-dimensional affinity electrophoresis

First-dimension electrophoresis

PAG-IEF is carried out with capillary gel (T = 5%, C = 3%[a]; carrier ampholite: pH 4–9, 1.25%, pH 5–8, 1.25%) prepared in a glass capillary tube (85 mm × 1.25 mm I.D.), using a thermostated disc electrophoresis apparatus (see Fig. 1).

Preparation of capillary PAG (see Fig. 1 and Table I). Prepare the working gel solution just before the gel preparation as shown in Table I. Prepare the IEF gel solution (solution A: 5.867 ml) and ammonium peroxodisulphate solution (10 ml) separately. Deaerate both solutions with suction by an aspirator for 15–20 min. Add 0.143 ml of the ammonium peroxodisulphate solution and 6 μl of TEMED to solution A and mix well. Pour about two thirds of the solution into a plastic cylinder (Fig. 1a: 80 mm high × 12 mm diameter) and immerse several clean glass capillaries carefully in the cylinder, avoiding the formation of air bubbles. Pour the residual portion of the working gel solution carefully into the cylinder until the meniscus of the solution in the capillaries reaches just 75 mm high. At this moment, the top of the solution in the cylinder is about 5 mm lower than the meniscus in the capillaries. Water is not overlayed on the top of the gel solution in the capillaries. After allowing the cylinder to stand at room temperature about for 10 min, a new surface of PAG is formed 0.5–0.7 mm beneath the original meniscus of the gel solution. When the capillaries are shielded carefully to avoid drying up and stored at 5–7°C, they can be used for several weeks without any problems in electrophoresis.

Attachment of the IEF-PAG capillary tubes to the electrophoresis apparatus (see Fig. 1b). Prepare the upper electrode (anodic) solution (0.04 *M* L-glutamic acid, prepared by dissolving 5.89 g of L-glutamic acid in water and diluting to 1 l and the lower electrode (cathodic) solution (1 *M* NaOH). Prepare the sample solution by mixing 0.027 ml of anti-DNP antibody solution (0.75 mg ml^{-1}) with 0.055 ml of PBS and dissolving 0.03 g of sucrose in it (total 0.1 ml). A 10-μl volume of the sample solution contains 2 μg of the antibody. The mixing composition depends on the concentration of the antibody solution. In a standard experiment, 1–2 μg of antibody (5–10 μl) were used for one capillary tube.

In Fig. 1b, the thermostated disc gel electrophoresis apparatus is shown. Pour the lower electrode solution (about 330 ml) into the lower electrode vessel (V_l) and cool to 4°C by circulating cold water through the glass condenser (C).

Remove the capillary tubes carefully one by one from the cylinder and wipe up gel crumbs clinging to the outer wall of the tubes with a clean tissue-paper. Top up the tubes with the upper electrode buffer using a small syringe, and overlay 5–10 μl of the sample solution beneath the electrode solution using a 25-μl micropipette, setting the point of the needle about 1 mm above the gel surface. Attach the capillary tubes to the upper electrode vessel (V_u) with a rubber connector (R). Place the upper elec-

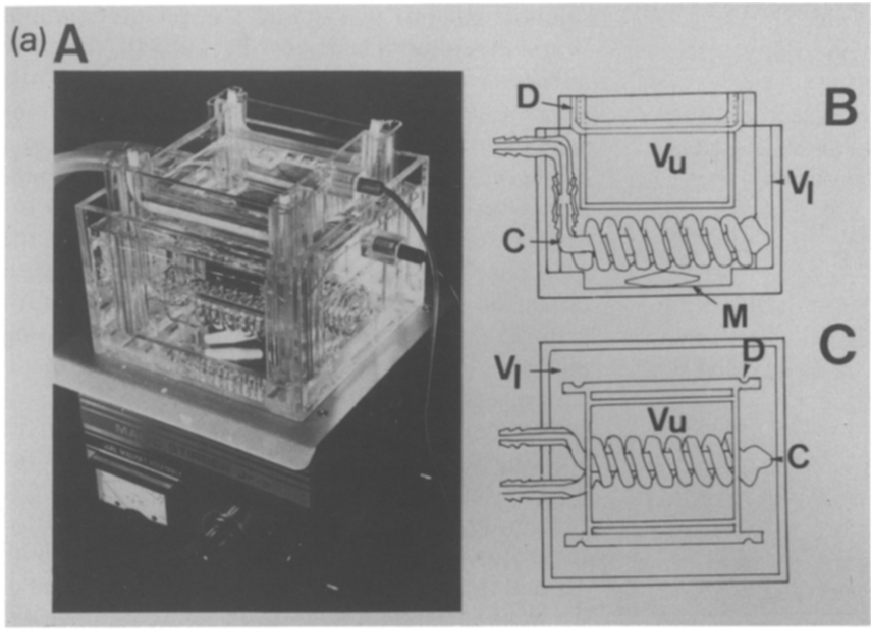
TABLE I

PREPARATION OF WORKING SOLUTIONS FOR CAPILLARY IEF-PAG

Stock solution or reagents[a]		Volume or amount
Solution A:		
Acrylamide solution:		0.6 ml
Acrylamide 48.5 g		
Bis 1.5 g		
Water to 100 ml		
Carrier ampholite:		
Pharmalite, pH range	4–6.5	0.094 ml
	6.5–9	0.094 ml
	5–8	0.188 ml
Glycerol solution: 40% (w/v)		1.5 ml
L-Lysine solution:		0.8 ml
L-Lysine 1.58 g		
Water to 100 ml		
Water		0.98 ml
Urea		2.162 g
Ammonium peroxodisulphate solution (freshly prepared)		0.143 ml
Ammonium peroxodishulphate 0.21 g		
Water to 10 ml		
TEMED		6 μl
Total		6.0 ml

[a] Solution A and ammonium peroxodisulphate solution are prepared separately just before the preparation of gel and deaerated *in vacuo* for 15 min, mixed carefully and finally TEMED is added to the mixed solution.

[a] C = g N,N′-methylenebisacrylamide (Bis)/%T; T = g acrylamide + g Bis per 100 ml of solution.



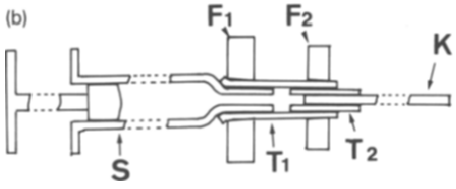


Fig. 2. (a) (A) Photograph and (B and C) diagrams of thermostated semimicro polyacrylamide gel slab electrophoresis apparatus. C = Glass condenser; D = connector for slab electrophoresis cell; M = magnetic stirrer; V_l and V_u = lower and upper electrode vessels. (b) Diagram of gel expeller. F_1 and F_2 = plastic rings (large and small); K = IEF capillary tube; S = plastic syringe (1-ml scale); T_1 and T_2 = plastic tubes.

trode vessel on the lower vessel (V_l) and set the condenser in the middle of the apparatus. Remove air bubbles from the bottom of the tube carefully with a syringe. If air remains under the upper electrode vessel, fill the lower vessel with the lower electrode solution and attach a thermometer (T) and, if necessary, rubber stoppers to the vessel. Pour 30 ml of the upper electrode solution gently into the upper vessel, avoiding disturbances to the layer of the sample solution.

Electrophoresis. Electrophoresis is carried out at 4°C using a constant-voltage supplier with stepwise elevation of the voltage from 250 V for 15 min, to 500 V for 15 min, 1000 V for 15 min and 2000 V for 2 h. If the sample antibody solution has a much higher ionic strength than that of PBS, the last step of the electrophoresis could be lengthened for an additional 20–30 min. Detach the capillary tubes from the apparatus to be subjected to the second electrophoresis.

Second-dimension electrophoresis: slab PAG affinity electrophoresis

AEP is carried out with the modified discontinuous buffer system reported by Reisfeld *et al.* [7], using a thermostated semimicro polyacrylamide slab gel electrophoresis apparatus. Two slab gel plates (inner space 100 mm high × 85 mm wide × 1 mm thick) can be attached to the apparatus (see Fig. 2a).

Preparation of slab PAG (see Fig. 2 and Tables II–IV. The working solutions of slab PAG for a

TABLE II

PREPARATION OF SEPARATING GEL (T = 5.13%, C = 2.59%; pH 4.3)

Stock solution[a]	Volume of working gel solution (ml) for one sheet of	
	Control gel	Affinity gel
Solution B:		
Acrylamide solution: Acrylamide 40 g, Bis 1.067 g, Water to 100 ml	1.0	1.0
Buffer solution, pH 4.3: 1 *M* KOH 48 ml, Acetic acid 17.2 ml, TEMED 4.0 ml, Water to 100 ml	2.0	2.0
DNP-PA solution: Any concentration[b]	–	2.73
Water	2.73	–
Sucrose solution 40% (w/v)	2.0	2.0
Ammonium peroxodisulphate solution: Ammonium peroxodisulphate 0.21 g, Water to 10 ml	0.27	0.27

[a] All stock solutions should be prepared using ultra-pure water. We used water prepared with a Milli-Q Plus Ultra-Pure Water System (Millipore).

[b] Separating gel for the affinity gels are prepared as the gel containing DNP-PA generally from 50 to 500 μM, depending on the affinity of anti-DNP antibody to DNP-hapten. The necessary volume of DNP-PA stock solution is adjusted with water.

control gel and for AEP gels are prepared according to Tables II and III. In general, four sets of slab gel plates are assembled, one for the control gel and three for the affinity gel containing various concentrations of DNP-PA, depending on the affinity of antibodies to DNP-hapten. The working solution is prepared for each gel.

Attach four sets of the slab gel plates to two sets of the upper electrode vessels, each of which is assembled with two sets of the slab gel plates, and place them in a incubator at 37°C. Prepare four sets of the working separating gel solutions (solution B, 7.73 ml in Table II) for one control gel and for three affinity gels containing various concentrations of DNP-PA in small beakers (50 ml) and store in the incubator. Prepare ammonium peroxodisulphate solution (10 ml) just before the gel preparation. Warm the solution in a water-bath at 37°C for a few minutes and add 0.27 ml of the solution to the working gel solution carefully. Mix the solutions gently and pour into one of the slab gel plates to a height of 70 mm, avoiding the formation of air bubbles. Overlay water on the top of the solution for the control gel or DNP-PA solution which has the same concentration of DNP-PA as in the affinity gel on the top of the solutions for the affinity gels with a syringe, avoiding disturbances to the gel solution meniscus, and stand upright in the incubator. Gelfication is completed in 30–40 min.

During the gelfication of the separating gels, the working solution for stacking gel is prepared according to Table III.

Remove the slab gel plate assemblies from the incubator, stand them on a table and wrap both sides of the affinity gel plates with aluminium foil to protect the DNP residue from light. Remove the

TABLE III

PREPARATION OF STACKING GEL (T = 3.13%, C = 20.0%; pH 6.7) AND STACKING SOLUTION (pH 6.7)

Stock solution[a]	Volume of solution (ml) for	
	Stacking gel	Stacking solution
Acrylamide solution: Acrylamide 10 g, Bis 2.5 g, Water to 100 ml	1.5	–
Buffer solution containing TEMED: 1 *M* KOH 48 ml, Acetic acid 2.87 ml, TEMED 0.46 ml, Water to 100 ml	1.5	–
Buffer solution without TEMED: 1 *M* KOH 48 ml, Acetic acid 2.87 ml, Water to 100 ml	–	3.0
Sucrose solution 40% (w/v)	1.5	1.5
Water	0.75	3.7
Methylene blue solution 0.05% (w/v)	–	3.8
Riboflavin solution 0.004% (w/v)	0.75	–
Total	6.0	12.0

[a] All stock solutions should be prepared using ultra-pure water (see Table II).

TABLE IV

ELECTRODE BUFFER SOLUTION FOR THE SECOND ELECTROPHORESIS

Stock solution[a]	Working solution
Stock buffer solution, pH 4.5: β-Alanine 31.2 g Acetic acid 8.0 ml Water to 1 l	1 volume
Water	4 volumes

[a] Stock solution should be prepared using ultra-pure water (see Table II).

overlayed solution with a syringe, wash the gel top once with the stacking gel solution, stand upright and overlay the stacking gel solution to a height of 10 mm. Illuminate the portion of the stacking gel solution with a fluorescent lamp. Gelfication is completed in 20–30 min.

Setting up IEF capillary gel for electrophoresis. Prepare 3 l of the electrode buffer according to Table IV and pour about 1 l of the buffer into the lower electrode vessel, circulate water at an arbitrarily constant temperature and stir with a magnetic stirrer to maintain the buffer at the desired temperature.

Attach the IEF capillary tube to an expeller, which has been filled with the stacking solution (Fig. 2b) at the end of the anodic side of the capillary, and first expel the capillary gel to a distance of *ca.* 1 cm. Wash the expelled portion of the capillary gel carefully with a small volume of water, then expel the gel completely, lay it carefully on top of the stacking gel, avoiding deformation of the gel, and make a good contact with the stacking gel. Overlay the stacking solution on top of the stacking gel to a height of 2 mm and place the upper electrode vessel to which is attached two sets of the slab gel plates on the lower electrode vessel, and carefully overlay the electrode buffer on the stacking solution until the buffer overflows slightly into the upper electrode vessel, avoiding disturbances to the meniscus of the stacking solution. Fill the upper electrode

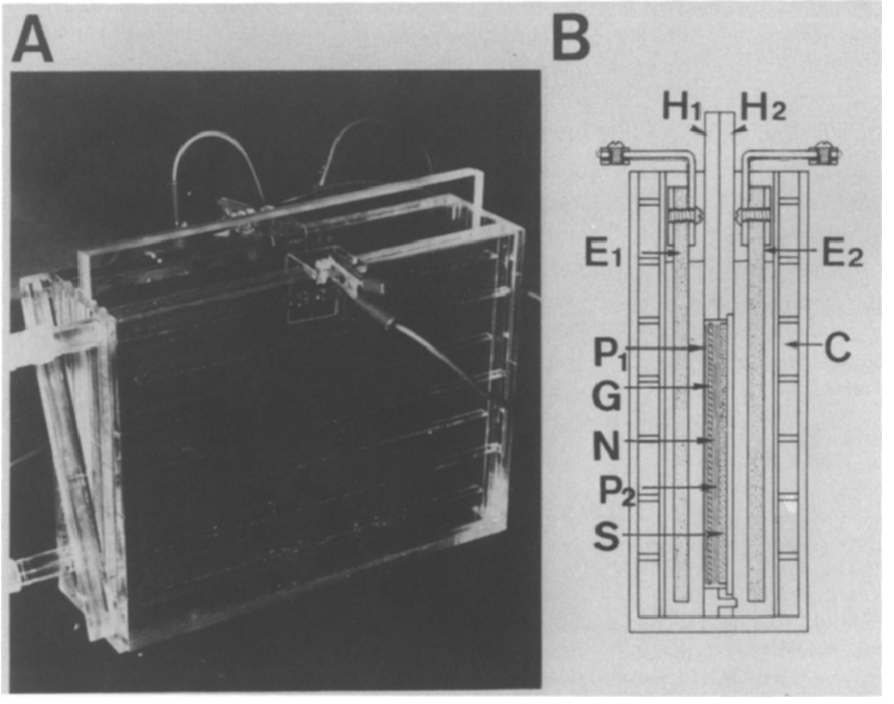


Fig. 3. (A) Photograph and (B) Cross-section of thermostated eletroblotting apparatus. C = Cooling plates; E_1 and E_2 = graphite plates, with platinum electrodes connected to the top of the plates; G = slab gel; N = nitrocellulose membrane; P_1 and P_2 = filter-papers.

TABLE V

ELECTRODE BUFFER FOR ELECTROBLOTTING

Stock solution	Working solution (50 m*M* glycine, pH 2.5)
1 *M* glycine: Glycine 75.07 g Water to 1 l	200 ml
1 *M* HCl	100 ml
Water	To 4 l

vessel with about 300 ml of the buffer. Commence electrophoresis when the temperature becomes constant, first at 50 V for 15 min and then at 80 V for 2.5–3 h, until the tracking dye (methylene blue) just reaches the bottom of the separating gel.

Detection of anti-hapten antibody

Electrophoretic transfer. Electrophoretic transfer is carried out using a thermostated electroblotting apparatus (see Fig. 3). A constant low temperature of 3°C prevents the formation of air bubbles during electroblotting. Electrophoresed slab gels are set in the gel holder, which is composed of two plastic frames (gel holder, H_1, and its cover, H_2) having two windows (85 × 102 mm) which are lined with Salan nets.

Dip four sheets of nitrocellulose (NC) membrane (Millipore, pore size 0.22 μm, 100 × 75 mm), eight sheets of Whatman 3MM filter-paper (100 × 80 mm) and two pieces of nylon sponge pad (100 × 85 mm; Scotch-Brite, 3M, St. Paul, MN, USA) in the blotting buffer (see Table V) and deaerate extensively with suction by an aspirator for 1 h. All procedures for electroblotting are performed at room temperature and surgical rubber gloves should be worn on both hands.

Dip the plastic gel holder (H_1) into the blotting buffer in a developing dish (32 × 23 × 6 cm). Place on each window one sheet of the deaerated Whatman 3 MM filter-paper, and lay on it one sheet of NC membrane, avoiding leaving any air bubbles

TABLE VI

DETECTION PROCEDURE FOR ANTI-DNP ANTIBODY

No.	Step	Reagent or solution	Volume per NC membrane (ml)	Duration and conditions[a]
1	Washing	TBS[b]	100	Overnight
2	Blocking	Blocking solution[c]	20	1 h
3	Washing	TBS[b]	50	30 s
4	First Ab fixation	First Ab solution[d]	10	3 h
5	Washing	Tween–TBD[e]	100	10 min
	Repeat the washing 4 times			
6	Second Ab fixation	Second Ab solution[f]	10	2 h
7	Washing	Tween–TBS[e]	100	10 min
	Repeat the washing 4 times			
8	Peroxidase reaction	Peroxidase substrate solution[g]	30	5–15 min
9	Washing[h]	Water	100	2–10 min
	Repeat the washing 4 times			

[a] All steps of the procedure are performed at room temperature with gentle shaking.

[b] Tris-buffered saline (TBS): 2 *M* Tris 50 ml, 1 *M* HCl 81 ml, NaCl 43.5 g and water to 5 l.

[c] Blocking buffer solution: 5% skim-milk solution of TBS.

[d] First antibody solution: goat anti-rabbit IgG 0.08 ml, goat anti-rabbit IgM 0.04 ml and 1% skim-milk solution of TBS 40 ml.

[e] Tween–TBS: 0.05% Tween-20 solution of TBS.

[f] Second antibody solution: peroxidase-conjugated rabbit anti-goat IgG 0.02 ml and 1% skim-milk solution of TBS 40 ml.

[g] Peroxidase substrate solution: 4-chloro-1-naphthol 0.06 g, cold methanol 20 ml, TBS 100 ml and 30% H_2O_2 0.06 ml. Prepare the solution just before step 8.

[h] Incomplete washing results in smear formation of NC membrane after a period due to residual reagents. In the first and the second washings, water is exchanged every 2 min and then after 10 min.

between the filter-paper and the NC membrane. Remove the slab gel plate from the upper electrode vessel, remove the cover-plate and mark the number on the slab gel. We use a metal punch 2 mm in diameter, punching the corner of the anodic side of the bottom of the slag gel in the first electrophoresis from one to four pieces according to gels No. 1–4. Release the slab gel from the gel plates carefully using a metal spatula 85 mm wide. Dip the released slab gel into the water and lay it exactly rectangularly and carefully on the NC membrane, avoiding leaving any air bubbles between the gel and the

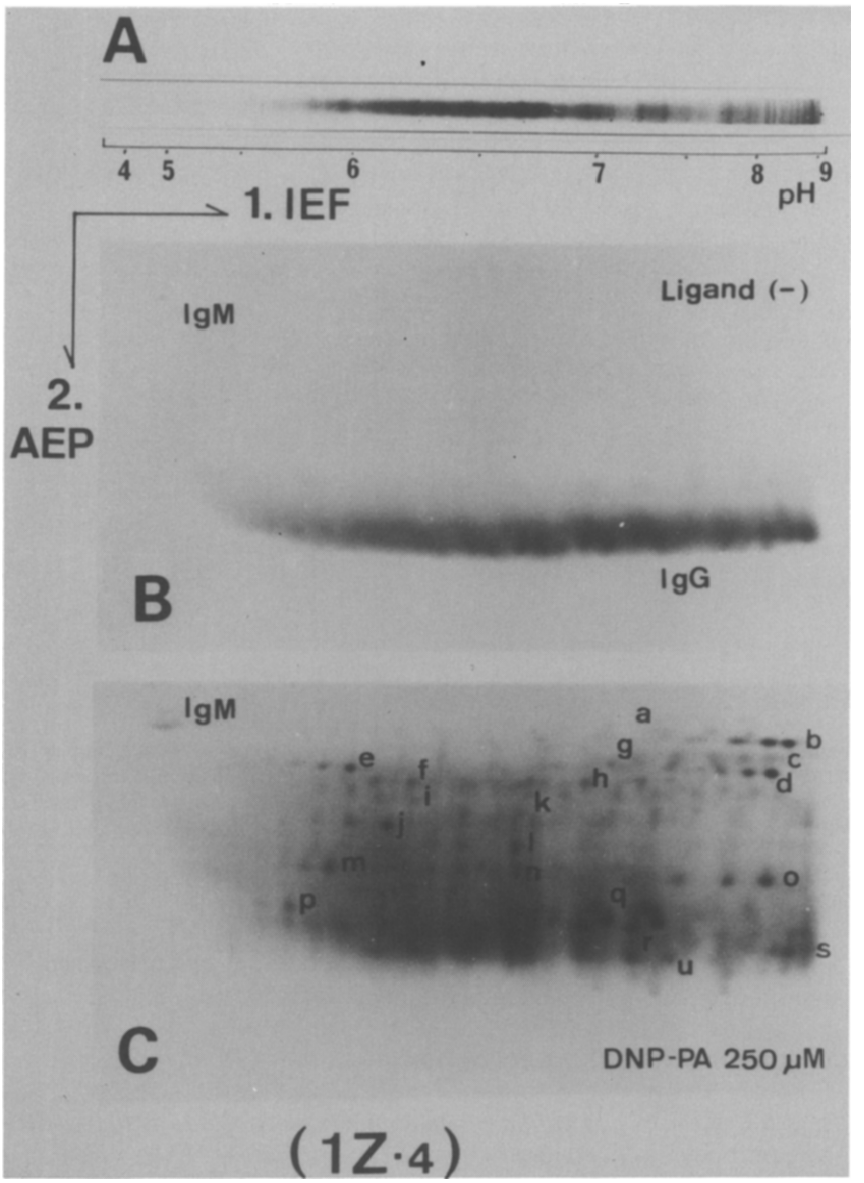


Fig. 4. Two-dimensional affinity electrophoresis patterns of anti-DNP antibody. (A) IEF pattern after staining with Coomassie BBG-250. Loading, 10 μg of antibody. (B) 2D-AEP pattern in the control gel (in the absence of DNP-PA). IgG class of anti-DNP antibody (IgG) migrates as a line and a trace of the IgM class of anti-DNP antibody (IgM) is detected as a spot in the corner of the gel. (C) 2D-AEP pattern in the affinity gel (in the presence of 250 μM DNP-PA). IgM is detected in the corner of the gel and IgG class of the antibody is spread over the entire gel. Clearly separated IgG spots are grouped into IgG families, designated a–u.

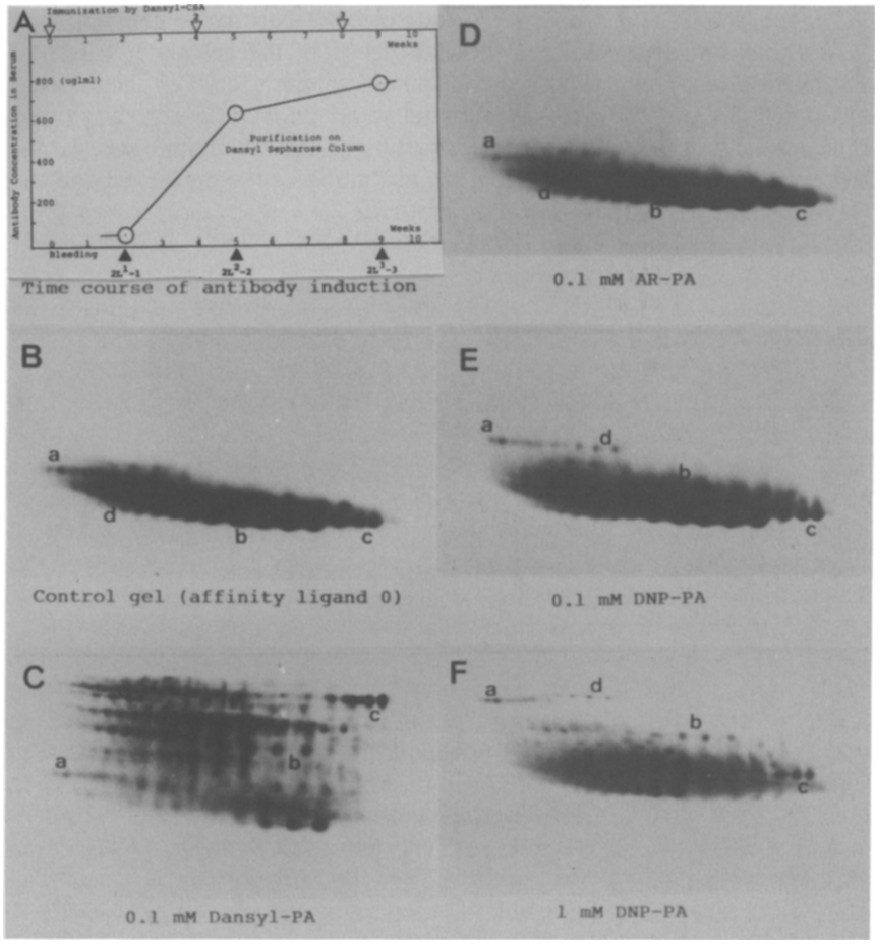


Fig. 5. Time course of antibody induction on immunization with dansyl-CSA and the 2D-AEP patterns of the anti-dansyl antibody to the homologous and heterologous haptens. (A) Time course of anti-dansyl antibody induction. Ordinate, antibody concentration (μg ml^{-1} serum). Abscissa, time (weeks). ▽ = Date of immunization; ▲ = date of bleeding. The antibodies were isolated and purified as indicated in the text. (B) 2D-AEP pattern of the antibody from the second bleeding in the control gel; (C) in the presence of 0.1 m*M* dansyl-PA; (D) 0.1 m*M* arsanilic diazotized (AR)-PA; (E) 0.1 m*M* DNP-PA; (F) 1 m*M* DNP-PA in the affinity gels.

NC membrane. Overlay another sheet of deaerated filter-paper and one sheet of deareated sponge pad, and cover with a plastic frame (H_2).

Pour the blotting buffer into the apparatus to cool the buffer prior setting the gel holder. Commence electrophoresis at 5 V for 2 h at 6°C.

Detection. Detection of anti-DNP antibody is performed by immunochemical fixation and thereafter by peroxidase activity staining. Each NC membrane is separately treated in a plastic Tupper ware vessel (10 × 14 × 3 cm) throughout the whole procedure. The procedure is summarized in Table VI. All steps of the procedure are performed at room temperature.

RESULTS AND DISCUSSION

Two-dimensional affinity electrophoresis (2D-AEP) pattern of anti-DNP antibodies [1,8]

In Fig. 4, 2D-AEP patterns of one of the rabbit anti-DNP antibodies are shown. Their p*I* distibution is shown in Fig. 4A. In the absence of the affinity ligand (with the control gel) (Fig. 4B, almost all

TABLE VII

CROSS-REACTIVITY OF ANTI-DANSYL ANTIBODY AND ITS APPARENT DISSOCIATION CONSTANTS (K_d^{app}) TO DANSYL- AND DNP-HAPTEN

Anti-dansyl IgG family	K_d^{app} [a] for		Affinity ratio (B/A)
	(A) Dansyl ($\times 10^4$ *M*)	(B) DNP ($\times 10^3$ *M*)	
a	4.77	0.296	0.62
b	0.846	0.364	4.30
c	0.276	1.47	53.4
d	0.353	0.161	4.55

[a] Affinity equation [11]: $1/r = (1/R_0)[1 + (c/K_d^{app})]$, where r = relative migration distance of protein in the presence of affinity ligand (in the affinity gel), R_0 = relative migration distance of protein in the absence of affinity ligand (in the control gel) and c = concentration of affinity ligand in the affinity gel.

of the antibody fractions migrated as a single line with a weak spot detected in the corner of the gel. The fractions correspond to the IgG class and the spot to the IgM class of anti-DNP immunoglobulins. In the presence of DNP-PA (with the affinity gel). (Fig. 4C), the antibody was separated into several hundred spots. The longer the migration distance, the weaker is the affinity to the DNP-hapten. In this unique distribution pattern, we could fractionate many families of IgG molecules, composed of several IgG spots migrating in a line, marked with letters a–u. The IgG spots belonging to the same family have the same affinity to the DNP-hapten but different p*I*s. From additional results obtained by cross-reactivity tests on the antibodies with DNP-, trinitrophenyl- and dansyl-haptens and by SDS-PAGE, we suggest that each family is derived from a clone of hapten-specific antibody producing cell lines.

Two-dimensional affinity electrophoresis pattern of anti-dansyl antibodies

Fig. 5C shows the 2D-AEP pattern of one of the anti-dansyl antibodies obtained with an affinity gel containing 100 μM dansyl-PA. Similarly to the anti-DNP antibody (see Fig. 4C), the anti-dansyl antibody is separated into several hundred IgG spots. They can also be grouped in many families which are composed of several IgG spots having the same affinity to the hapten but different p*I* values. They could be grouped into more than 40 IgG families and the clearly distinguished families are numbered alphabetically (a–d).

Cross-reactivity of anti-hapten antibodies [9,10]

Almost all of IgG families of rabbit anti-DNP antibodies have affinity to TNP-hapten [9]. In general, their affinity to DNP-hapten is higher than that to TNP-hapten. However, a few families have a higher affinity to TNP-hapten. In the latter case, the anti-DNP-IgG family migrates in the affinity gel containing TNP-PA in a line having the same affinity but different p*I* values.

Fig. 5F shows the 2D-AEP pattern of the same anti-dansyl antibody as mentioned above with affinity gel containing 1 m*M* DNP-PA [10]. A large portion of the antibody does not show affinity to DNP-hapten. However, some of them show affinity to it. The apparent dissociation constants (K_d^{app}) of the families to both haptens calculated according to the affinity theory are given in Table VII. The anti-dansyl IgG family a has 1.6-fold higher affinity to DNP-hapten, whereas families b, c and d have 4.3-, 53.4- and 4.55-fold higher affinities to the dansyl hapten than to the DNP-hapten. In all cases, every family of the anti-dansyl IgG migrates in both affinity gels in a line having the same affinity but different p*I* values.

REFERENCES

1 K. Takeo, R. Suzuno, T. Tanaka, and K. Nakamura, *Electrophoresis*, 10 (1989) 813–818.
2 K. Takeo, R. Suzuno, M. Fujimoto, T. Tanaka and A. Kuwahara, in D. Stathakos (Editor), *Electrophoresis '82*, Walter de Gruyter, Berlin, 1983, pp. 277–283.

3 K. Takeo, *Adv. Electrophoresis*, 1 (1987) 229–279.
4 R. R. Porter, *Methods Med. Res.*, 3 (1950) 256–271.
5 C. W. Parker, T. J. Yoo, M. C. Johnsor and S. M. Godt, *Biochemistry*, 6 (1967) 3408–3416.
6 H. N. Eisen, *Methods Med. Res.*, 10 (1964) 94-102.
7 R. A. Reisfeld, U. J. Lewis and D. E. Williams, *Nature (London)*, 195 (1962) 281–283.
8 K. Takeo, R. Suzuno, T. Tanaka, K. Nakamura and A. Kuwahara, in L. M. Dunn (Editor), *Electrophoresis '86*, Verlag Chemie, Weinheim, 1986, pp. 233-240.
9 K. Takeo, R. Suzuno, T. Tanaka, M. Fujimoto, A. Kuwahara and K. Nakamura, *Protides Biol. Fluids., Proc. Colloq.*, 32 (1984) 969–972.
10 K. Takeo, T. Tanaka, K. Nakamura and R. Suzuno, *Electrophoresis*, 10 (1989) 818–824.
11 T. C. Bøg-Hansen and K. Takeo, *Electrophoresis*, 1 (1980) 67–71.