Note

Affinity electrophoresis

I. Studies to optimise conditions

MICHEL CARON, ALAIN FAURE* and PIERRE CORNILLOT

Département de Biochimie, Faculté de Médicine de Bobigny, 74, rue Marcel Cachin, 93000-Bobigny (France)

In the last few years, there has been a rapid increase in the utilisation of highly specific interactions between complementary biological molecules by affinity chromatography. This use may be limited (especially for small quantities) by specific interactions, elution problems, etc., but a combination of the principles of electrophoresis and biospecific interaction may help to solve such problems. An immuno-affinoelectrophoresis technique has recently been described for use in predicting the result of affinity chromatography[1]. Various derivatives of Sepharose[2,3,4] have been tested and applied in affinity electrophoresis; this work is an approach to a better understanding of the interactions that occur during immuno-adsorption in a simple system.

MATERIALS AND METHODS

The basic equipment used for all experiments is an LKB 2117 Multiphor apparatus. The gels (1.2 % agarose; Indubiose A 37 L'Industrie Biologique Française) are cast on 12.5 × 9.4 cm glass plates. After electrophoresis, the plates are stained for 10 min in a solution of Amido Black 10B, then washed in 20 % aqueous acetic acid.

Affinity electrophoresis

The principles of the modifications from simple immuno-electrophoresis to affinity electrophoresis can be seen from Fig. 1. Gel 1 contains only agarose; anti-human serum albumin coupled to Sepharose (as described below) is added to gel 2 (in a volume ratio of 1:1); unsubstituted activated Sepharose to gel 3 (volume ratio 1:1); and free antibodies to gel 4 (0.12 ml of antibodies per ml of agarose). The samples are placed in the wells in the agarose gel, and the wells are filled with 5 μl of human serum albumin (HSA) solution (2 mg per ml). Ten samples can be run on each gel.

The migration of proteins is estimated by direct detection on the plates without free antibodies (see Fig. 2b); quantitative estimation of the proteins, after immuno-adsorption, is achieved by electrophoresis in agarose gel containing antibodies as shown in Fig. 2a (see also ref. 5).

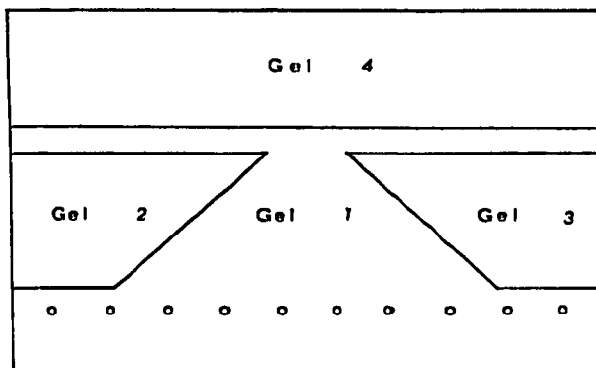* Present address: C.N.T.S., 6, rue A. Cabanel, 75015 Paris, France.

Fig. 1. Disposition of gels for affinity electrophoresis. Gel 1, agarose (total volume 21 ml); gel 2, agarose containing activated Sepharose (3 ml); gel 3, agarose containing anti-HSA coupled to Sepharose; gel 4, agarose containing free antibodies. For determination of the migration distance, gel 4 contains no antibodies.

Preparation of immunoadsorbents

Sepharose 4B (Pharmacia, Uppsala, Sweden), cyanogen bromide (Schuchart, München, G.F.R.) and antisera from the Centre Départemental de Transfusion Sanquine (Bois Guillaume), were used.

Antisera (0.1 ml per ml of Sepharose) are coupled to Sepharose activated with cyanogen bromide (200 mg per ml of Sepharose) by the method of Porath et al.[6,7] as modified by Cuatrecasas et al.[8]. After being gently stirred overnight at 4°, the suspension is washed with large volumes of buffer solutions of pH 8.6 (barbitone), pH 5 (sodium acetate) and pH 8.6. The amount of ligand bound to the adsorbent is calculated from the protein remaining after washing ($\approx 60\%$).

Buffer solutions

0.1 M Glycine–hydrochloric acid, pH 2.8.
0.5 M Acetate–acetic acid, pH 5.
0.12 M Phosphate, pH 7.
Barbitone, pH 8.6, ionic strength 0.075.
Carbonate–bicarbonate 0.1 M, pH 10.7.

Temperature

Efficient cooling is obtained in the Multiphor apparatus, as the cooling plate is made from glass. Refrigeration fluid comes from a cooling thermostat.

Speed of migration

Variations in speed of migration are obtained by varying the applied potential (from 4.5 to 13.5 volts per cm.

Reversibility of antigen–insolubilised antibodies complex

To investigate the possibility of separation between (anti-albumin)–Sepharose and adsorbed albumin, the immuno-adsorbent is prepared as described above, and affinity chromatography is performed as follows.
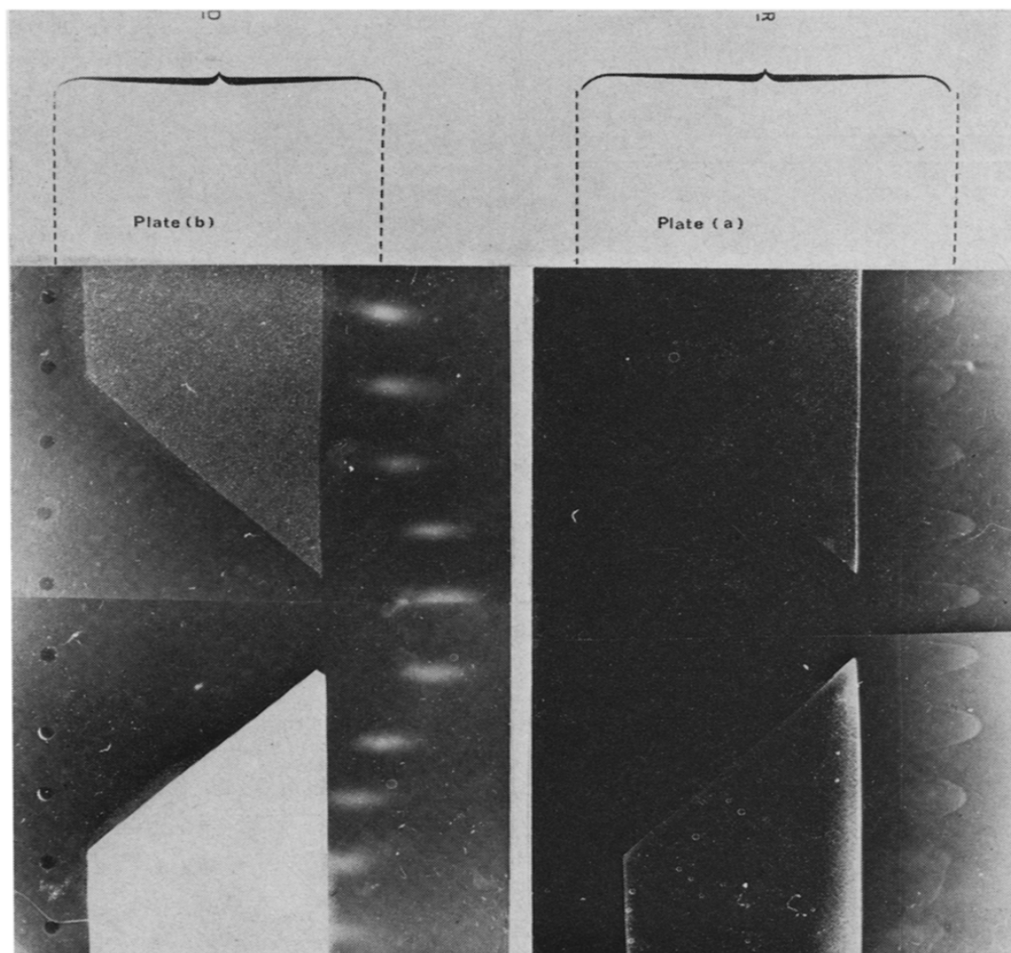
Fig. 2. Representation of the two types of plates. (a) With antibodies; estimation of antigens by quantitative precipitation (R = "rocket"). (b) Without antibodies; distance of migration (D). Experimental conditions: 10°; 10 V/cm; pH 8.6; 10 μg of albumin per well; migration for 4 h.

A column is filled with a gel of Sepharose 4B (10 ml) substituted with anti-HSA, and HSA (16 mg in 4 ml) is passed through the column. The immuno-adsorbent is washed with 0.5 M sodium chloride, 0.05 M Tris–HCl of pH 8 and barbitone buffer of pH 8.6 until the optical density of the eluate at 280 nm reaches a constant minimal value. The gel is then removed from the column and used to cast a plate as seen in Fig. 3. Electrophoresis on such a plate is performed at 10 V/cm for 200 min, and the plate is stained as described above.

RESULTS AND DISCUSSION

Choice of pH

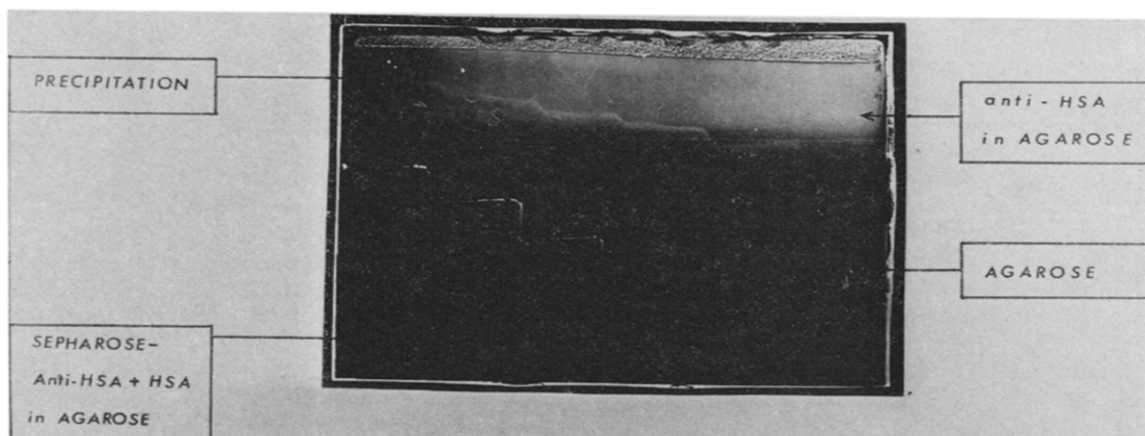During these experiments, the affinity electrophoresis was run for 4 h at 9 V/cm

Fig. 3. Dissociation of HSA from Sepharose–anti-HSA in an electric field. Experimental conditions: 10°; 10 V/cm; pH 8.6; gel, agarose plus anti-HSA (6.5 ml of agarose and 0.7 ml of anti-HSA).

(25 to 30 mA); the direction of migration was detected by cellulose acetate electrophoresis.

The use of pH 2.8 and pH 10.7 was incompatible with the production of "rockets" (antigen–antibody precipitates).

At pH 5 there was slow migration of HSA, with slight retention during passage through the immuno-adsorbent. The slowness of migration made this pH unsuitable.

At pH 7 and 8.6 (the most useful pH values), there was good diminution of migration distance and "rockets" (see Table I). The most important retention of HSA on Sepharose–anti-HSA was at pH 8.6 (the diminution in migration distance was 19 % at pH 7 and 36 % at pH 8.6). Subsequent experiments were therefore performed at pH 8.6.

TABLE I

EFFECT OF pH

These results are for migration distances (in cm) in Sepharose–anti-HSA of 3.5 cm at 10° and 9 V/cm.

	pH	Without Sepharose (A)	With Sepharose (B)	With Sepharose–antibodies (C)	B/A	C/A	C/B
Distances							
	7	5.20	4.95	4.00	0.95	0.76	0.81
	8.6	6.65	6.25	4.00	0.94	0.60	0.64
"Rockets"							
	7	6.2	5.90	5.25	0.95	0.84	0.89
	8.6	5.75	5.40	4.90	0.94	0.85	0.90

Temperature

Temperature mainly affects the stability of the gel, although it may also be compatible with the specific interactions. We have used the following temperatures: 1°, 4°, 10°, 15°, 25° and 37°. At lower temperatures, small cracks appeared in the gel, and the retention of HSA was not apparent. At higher temperatures, there was a desiccation of the agarose. A constant temperature of 10° was chosen.

TABLE II

EFFECT OF VOLTAGE

Same conditions as in Fig. 1; pH 8.6, 10°.

	Voltage (V/cm)	Without Sepharose (A)	With Sepharose (B)	With Sepharose–antibodies (C)	B/A	C/A	C/B
Distances							
	6.5	4.75	4.30	3.85	0.91	0.81	0.89
	8	5.30	4.90	3.70	0.92	0.70	0.75
	9	6.65	6.25	4.00	0.94	0.60	0.64
	10.5	5.94	5.70	5.30	0.96	0.88	0.92
"Rockets"							
	9	5.75	5.40	4.90	0.95	0.86	0.89
	10.5	5.95	5.80	5.70	0.98	0.95	0.97

Speed of migration (voltage)

The choice of high or low voltage is important because it can affect electro-osmosis, the electro-osmotic flow being approximately proportional to the potential gradient[9]; it can also affect the gels, as high voltage damages gels (especially those containing Sepharose). We tested use of the following voltages: 4.5, 6.5, 8, 9, 10.5 and 13 V/cm with gels containing 1.2% of agarose (this concentration was necessary to ensure solidity of the gel during migration and handling.

At 4.5 or 6.5 V/cm, migration of HSA was poor. Consequently, after passage through immuno-adsorbent the 'rockets' were wider and the precision was less good.

At 10.5 or 13 V/cm damage often became visible on the gel (and between different parts of the gel during electrophoresis).

Better results were obtained with a potential of 8–9 V/cm (see Table II). The general conditions for obtaining good results in affinity electrophoresis are summarized in Table III.

TABLE III

EXPERIMENTAL CONDITIONS ESTABLISHED IN INVESTIGATIONS

Parameter	Value
pH	8.6
Temperature, °C	10
Potential gradient, V/cm	10
Current, mA	<30
Time, h	4
Distance between 2 wells, cm	At least 1.2
Agarose concentration, %	1.2
Amount of HSA per well, μg	10

Reversibility

The effects of an electric field on the Sepharose–anti-HSA gel after chromatographic adsorption of HSA are seen in Fig. 3; separation of at least part of the adsorbed albumin can be observed. This fact is suggested by the presence of a single precipitation line against anti-HSA. The same experiment with anti-rabbit γ-globulin

gave no line of precipitation. These results suggest the liberation of free albumin, and permit an understanding of Fig. 2; HSA is retarded, but not retained, by the solid phase. This explains why there is a larger difference between results with substituted and unsubstituted Sepharose for the distances of migration than for the "rockets" (zones of quantitative precipitation). For the same reasons, irregularities and sometimes superpositions of "rockets", can be observed after passage through the immuno-adsorbent. In his work on the fixation of serum glycoproteins on concanavalin A, Bøg-Hansen[1] obtained specific interaction without indication of reversibility.

This technique can be used in two ways: (1) in a single-step operation, involving sorption of molecules during migration, and (2) in a two-step operation, the first step being chromatography, involving dissociation of the couples obtained during the chromatographic step. The one-step operation is attractive because of its simplicity, as it permits study of an affinity reaction with only a few microlitres of product, and also allows us to obtain, in the same step, an immunological analysis of the biological materials concerned after specific adsorption. The two-step process offers an alternative to elution with denaturing agents. As with the one-step process, immunological determination of non-adsorbed molecules is possible.

ACKNOWLEDGEMENT

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