

**DOMUZ UTERUS SİTOZOLÜNDEN SİTOPLASMİK PROTEİNLERİN
İMMUNOKİMYASAL İZOLASYONU***

**IMMUNOCHEMICAL ISOLATION OF CYTOPLASMIC PROTEINS
FROM PIG UTERUS CYTOSOL**

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SUMMARY

Gamma globulin was isolated from goat anti-pig serum and coupled to CNBr activated Sepharose and serum proteins from pig uterus cytosol was separated using this immunoadsorbent.

ÖZET

Domuz serumu ile immunize edilen keçi serumundan gamma globulin izole edildi ve CNBr ile aktive edilen Sepharose'a kaplandı. Domuz uterus sitozolünün ihtiva ettiği serum proteinler bu immunoadsorbent kullanılarak ayrıldı.

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INTRODUCTION

Cytosol of pig uterus contains a lot of plasma proteins. The aim of this work was isolation of cytoplasmic proteins from pig uterus cytosol using an immunoadsorbent prepared by conjugating goat serum (against pig serum) to Sepharose CL-4B. The cytosol was applied on the column of antibody-coupled Sepharose in order to bind the contaminating plasma proteins. The effluent was examined for plasma proteins by immunoelectrophoresis, double-diffusion and radial immunodiffusion techniques. These processes were repeated until there were no detectable plasma proteins in the effluent, which was dialyzed against distilled water and lyophilized. Desorption of immunoadsorbent was achieved by several methods.

MATERIALS AND METHODS

Goat serum was prepared against pig serum. Sepharose CL-4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Cyanogen bromide and other chemicals were obtained from E. Merck (Darmstadt, Germany).

Immunoelectrophoresis: 2% agar-purum in Michealis buffer, water (1:1, V:V), 50 mA/Plate, 165 minutes, at 4°C were used.

Double immunodiffusion: 2% agar-purum in Michealis buffer, water (1:1, V:V), 5 µl sample, 20 µl antibody were used.

Single radial immunodiffusion: The processes were carried out as previously described (1). The agar contained goat serum against pig serum. The antigen wells were filled with serial dilutions of pig serum proteins or cytosol or cytoplasmic proteins of pig uterus.

Protein assay: Protein determinations were performed by the Bio-Rad protein assay (2, 3). Turbid samples were dissolved with Triton X-100 before protein assay treatment.

Preparation of Cytosol: All operations were carried out at 4°C. Immature pig uteri were collected at the local slaughterhouse as soon as the animals were killed, and kept in plastic bags buried in crushed ice while being rushed to the laboratory. The uteri were stripped off from connective tissue and coarsely minced in a meat grinder and suspended in 2 volumes of 10 mM sodium

phosphate buffer containing 150 mM NaCl and 5 mM NaN_3 pH 7.4 (PBS) and homogenized with an ultraturrax (Jahnke Kunkel TP18/10) for 10×10 seconds with intermittent pauses of 20 seconds. The homogenate was centrifuged for 90 minutes at 40,000 rpm (284,000 g) in a SW 40 rotor (Beckman Ultracentrifuge).

Isolation of gamma globulins from goat anti-sera against pig serum: This was carried out according to the method of Kekwick (4). Addition of 18 g/100 ml of Na_2SO_4 to 600 ml of goat serum against pig serum precipitated the globulins almost completely. The mixture was centrifuged at room temperature for 60 minutes, at 30,000 rpm in SW 40 rotor. The precipitate was dissolved in 0.1 M NaHCO_3 buffer (pH 8.5), made up to 40% of the initial serum volume, then was precipitated with Na_2SO_4 (12 g/100 ml) and centrifuged again. This precipitate was dissolved in 35% of the initial serum volume with 0.1 M NaHCO_3 (pH 8.5) then dialyzed against 0.1 M NaHCO_3 (pH 8.5) at room temperature for 16 hours.

Preparation of immunoadsorbent: 1200 ml of Sepharose CL-4B was washed twice with distilled water then with 0.5 M NaCl and again with distilled water and filtered over a Buchner funnel. The washed Sepharose was mixed with an equal volume of water and finally cyanogen bromide (300 mg/ml of packed sepharose) was added at once to the stirring suspension. The pH of the suspension was immediately raised to and maintained at 10-11 with 8M NaOH. The temperature is maintained at 18-22°C by adding pieces of ice as needed. The reaction was completed in 30 minutes as indicated by the cessation of proton release. A large amount of ice was rapidly added to the suspension, transferred quickly to a Buchner funnel. Activated Sepharose was washed with 20 times of its original volume and added to a plastic beaker containing 1200 ml of gamma globulin (5.8 mg/ml) of goat anti-pig serum. pH was adjusted to 8.5. The reaction mixture was gently stirred for 48 hours in the cold room. Immunoadsorbent was filtered with a Buchner funnel, washed with 3 volumes of 1 M ethanolamin (216 ml of ethanolamin + 3024 ml of water, adjusted to pH 8 with HCl) for 2 hours at room temperature. Immunoadsorbent was filtered and washed with PBS. Unbound protein was estimated in the filtrates of the immunoadsorbent obtained from buffer and ethanolamin.

RESULTS AND DISCUSSION

As shown in Table I, Kekwick preparation contained just over 20% of all proteins in goat antisera and the binding of gamma globulin to Sepharose CL-4B was nearly complete.

Table I. Synopsis of protein in the processes

	total volume (ml)	The concen- tration of protein (mg/ml)	total protein (mg)	%
Goat anti-pig serum	600	54.5	32 700	100
Kekwick Preparation	210	33.1	6 951	21.2
The washing of immuno- adsorbent after coupling				
a) with PBS	3 600	0.04	144	2
b) with ethanolamin	3 600	0.005	18	0.25
Bound protein (immuno-adsorbent)	1 200	5.65	6 789	97.66

The binding capacity of immuno-adsorbent: 0.4 ml of immuno-adsorbent or Sepharose CL-4B (control) were incubated with variable amounts of cytosol containing 7.8 mg/ml protein for 12 hours at 4°C (gentle stirring with magnetic stirrer). The suspension was centrifuged in an Eppendorf centrifuge and the amount of protein in the supernatant was measured. The results are shown in Table II.

Table II. Binding of cytosol protein to immuno-adsorbent

Immuno-adsor- bent or sepha- rose CL-4B (ml)	Volume of cytosol (ml)	Volume of buffer (ml)	The amount of protein in the supernatant		Binding to immuno- adsor- bent (%)
			Control (mg)	Immuno-adsorbent (mg)	
0.4	10	0	78	75.9	2.10
0.4	8	2	62.4	60.3	3.30
0.4	6	4	46.8	42.7	8.80
0.4	4	6	31.2	28	10.25
0.4	1	9	6.9	5.6	18.84
0.4	0.5	9.5	3.3	2.4	27.27
0.4	0.25	9.75	1.5	0.875	41.66
0.4	0.125	9.875	0.967	0.213	78.00

Fig. I. shows the binding of cytosol protein of pig uterus to immunoadsorbent. It's clear that a lot of cytoplasmic proteins bind to immunoadsorbent unspecifically because the amount of binding protein was too high. NaCl was used against unspecific binding to immunoadsorbent.

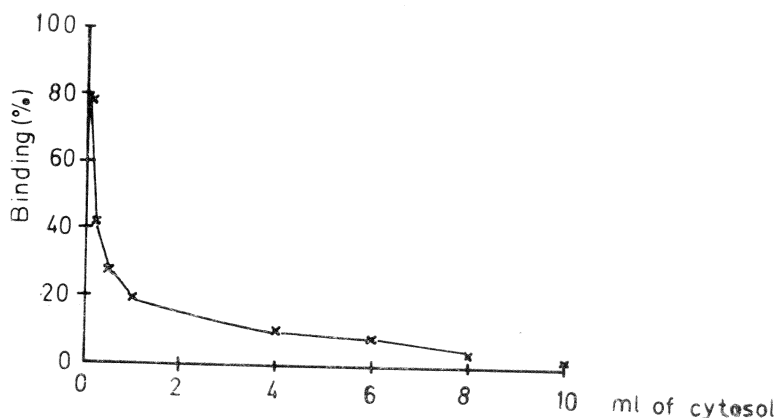
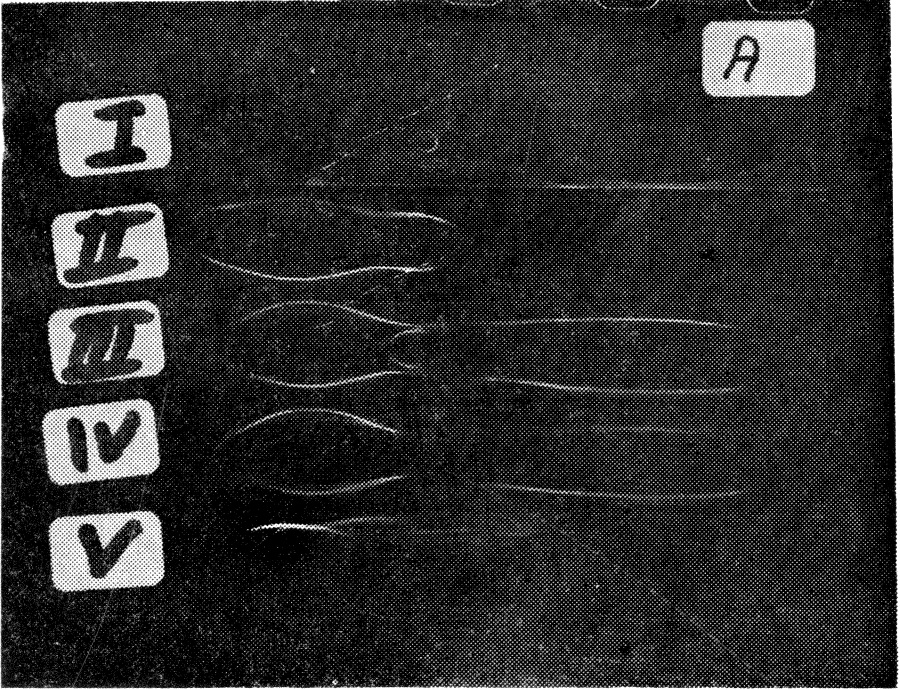


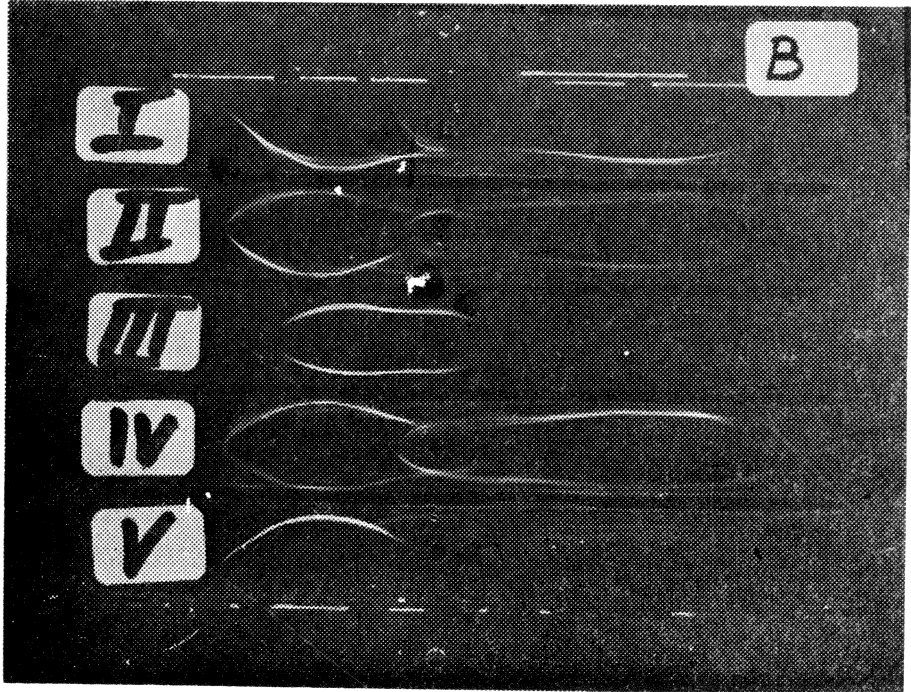
Fig. I. The binding of cytosol of pig uterus to immunoadsorbent.

Determination of amount of immunoadsorbent required for complete separation of plasma proteins from pig uterus cytosol containing 7.8 mg/ml protein: 100 ml of immunoadsorbent was incubated with variable amounts of cytosol (250, 125, 62.5, 31.2, 7 ml) in a total volume of 250 ml diluted with PBS. When necessary, the mixture was centrifuged and the supernatant was dialyzed against distilled water, lyophilized and examined for plasma proteins. The sample for immunoelectrophoresis contained 80-120 mg/ml of protein. As shown in photograph A.IV, at the ratio of 250 ml of cytosol to 100 ml of immunoadsorbent, the supernatant still shows a clear plasma protein band. However the haemopexin band is not there after immunoadsorption. When using 125 ml of the cytosol, the immunoglobulin band is weak (Photograph B, V); at 62.5 ml cytosol the immunoglobulin band disappears (Photograph B, III); at 31.2 ml of cytosol, plasma protein band is very weak (Photograph C, I) and finally if 7 ml of cytosol used, the supernatant shows no plasma protein band (Photograph D, IV).



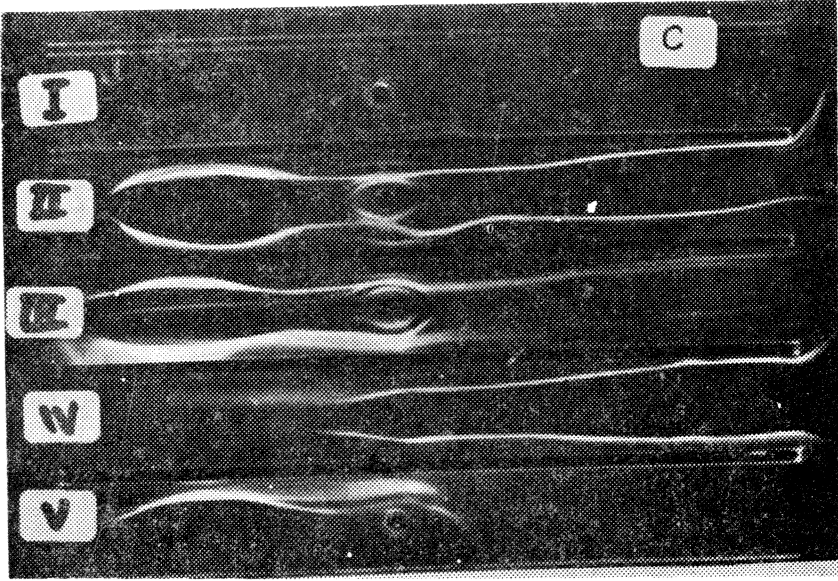
Photograph A: Immunoelectrophoresis of supernatant of antiplasma Sepharose

- I: Pig IgG (40 mg/ml).
- II: Pig albumin (6 mg/ml).
- III: Cytosol (120 mg/ml).
- IV: The supernatant of antiplasma Sepharose (120 mg/ml). (100 ml of immunoadsorbent + 250 ml of cytosol).
- V: Tryptic fragment (120 mg/ml).



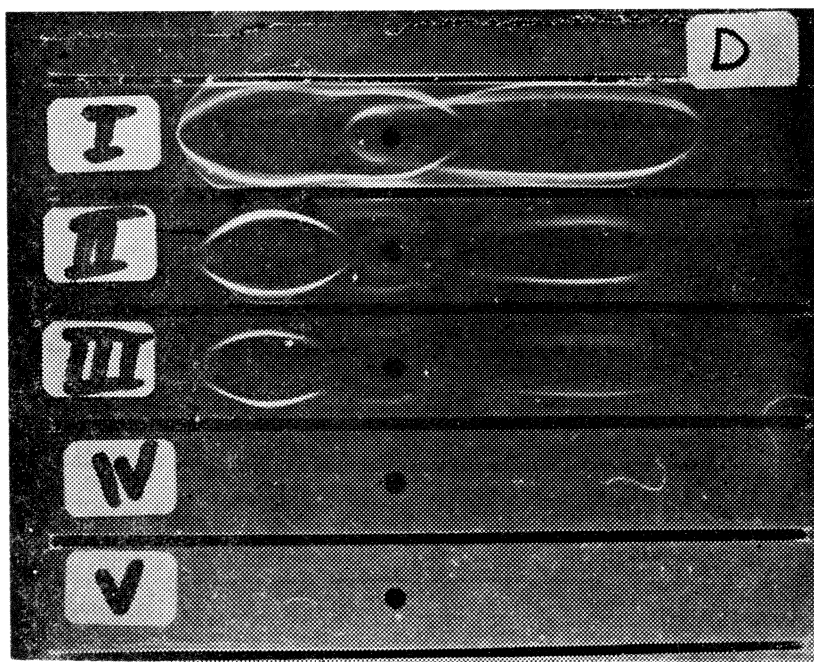
Photograph B: Immunoelectrophoresis of supernatant of antiplasma Sepharose

- I: Cytosol (120 mg/ml).
- II: , ,
- III: The supernatant of antiplasma Sepharose (120 mg/ml) (100 ml of immunoabsorbent + 62.5 ml of cytosol).
- IV: Cytosol (120 mg/ml).
- V: The supernatant of antiplasma sepharose (120 mg/ml). (100 ml of immunoabsorbent + 125 ml of cytosol).



Photograph C: Immunoelectrophoresis of supernatant of antiplasma Sepharose

- I: The supernatant of antiplasma Sepharose (80 mg/ml). (100 ml of immunoadsorbent + 31.2 ml of cytosol)
- II: Cytosol (120 mg/ml).
- III: Pig serum (55 mg/ml).
- IV: Pig IgG (40 mg/ml)
- V: Pig albumin (60 mg/ml)



Photograph D: Immunoelectrophoresis of supernatant of antiplasma Sepharose

- I: Cytosol (120 mg/ml).
- II: Cytosol (15 mg/ml).
- III: Cytosol (7.5 mg/ml).
- IV: The supernatant of antiplasma Sepharose (80 mg/ml).
(100 ml of immunoabsorbent + 7 ml of cytosol).
- V: The supernatant of antiplasma Sepharose (80 mg/ml).
(100 ml of immunoabsorbent + 7 ml of cytosol).

Separation of plasma protein from pig uterus cytosol on the immunoabsorbent column: 605 ml of the immunoabsorbent was packed in a wide column, washed with 10 mM Na phosphate buffer containing 0.5 M NaCl, 5 mM NaN_3 , (pH 7.4). 104 ml of cytosol (protein concentration 14.76 mg/ml) was then applied on the column, at a constant flow of 70 ml/hour. After cytosol application the column was washed with 10 mM Na phosphate, 0.5 M NaCl, 5 mM NaN_3 (pH 7.4.). The fractions containing protein

peaks were pooled and examined for plasma proteins. A solution of 8 M urea and 50 mM ethanolamin in PBS was used for desorption (Table II).

Table II. The separation of plasma proteins from pig uterus cytosol on the immunoadsorbent column

	volume	protein concentration (mg/ml)	total protein (mg)	% of initial protein	presence of plasma proteins
CYTOSOL	104	14.76	1535	100	all plasma proteins
EFFLUENT I	340	2.80	925	62	a few proteins were lost at starting point
» II	370	1.84	680	44	immunoglobulins were lost
» III	435	1.00	435	28	
» IV	482	0.51	245	16	immunolectrophoresis doesn't show any plasma proteins
» V	150	0.80	120	7.8	single immunodiffusion doesn't show any plasma proteins

This process was done 4 times (Fig. 2). 4. effluent of immunoadsorbent didn't show any plasma proteins with immunoelectrophoresis (Photograph E) but it showed with single radial immunodiffusion technique.

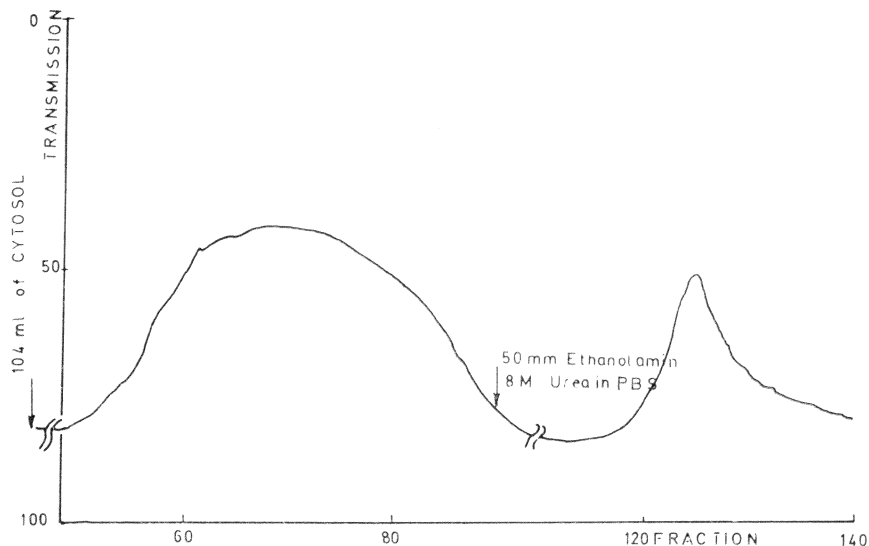
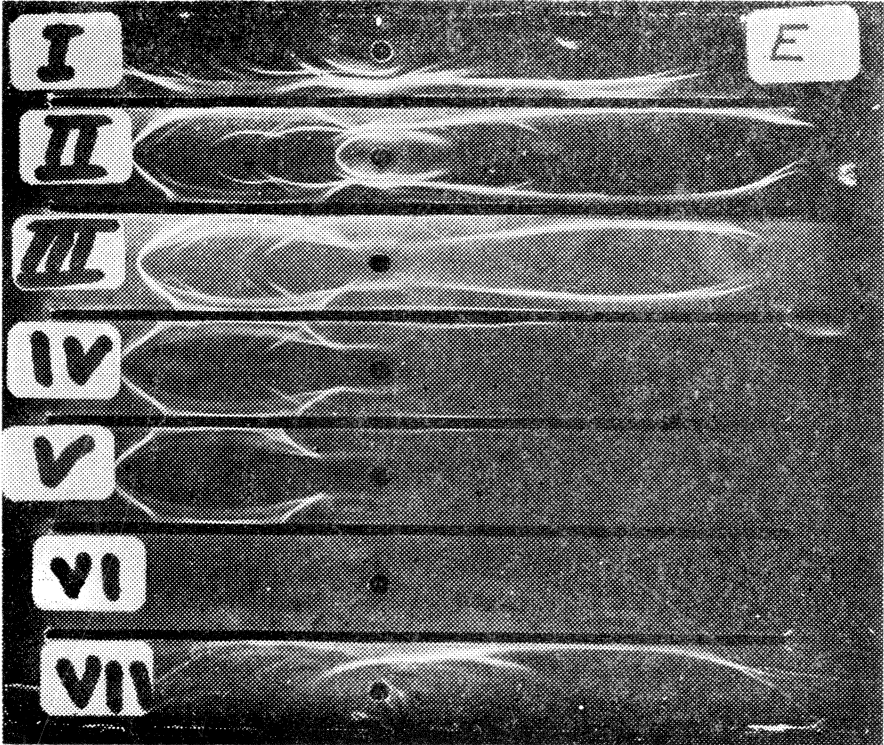


Fig. II. The separation of plasma proteins from pig uterus cytosol on the immunoadsorbent column and desorption of the column.

The V. effluent was prepared as follows: 245 mg of effluent IV was dissolved in 100 ml of 10 mM Na phosphate, 1 M NaCl, 5 mM NaN_3 , pH 7.4 and mixed with 30 ml of immunoadsorbent, stirred with magnetic stirrer for 12 hrs at 4°C . The suspension was filtered on a Buchner funnel and immunoadsorbent was washed with 50 ml of the above mentioned buffer and both filtrates were combined, dialyzed against distilled water, lyophilized and examined with single immunodiffusion technique (Photograph F, G). Effluent V. didn't show any plasma proteins.

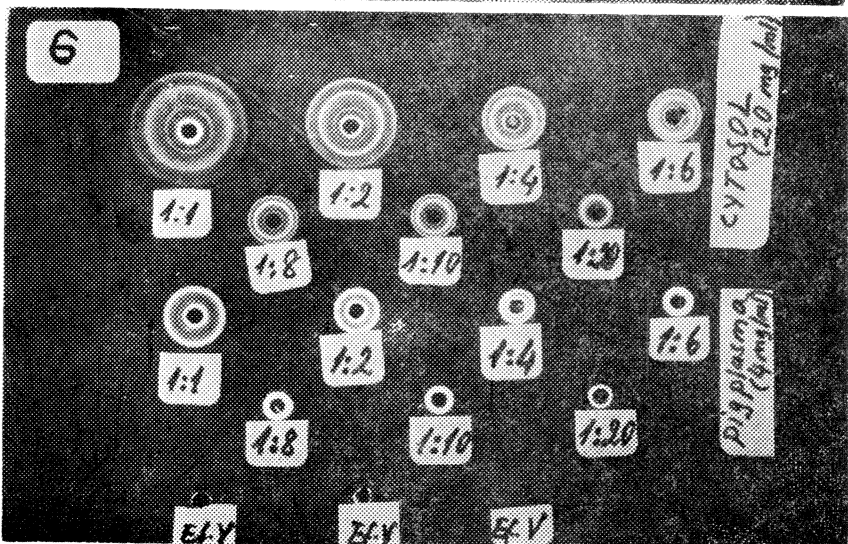
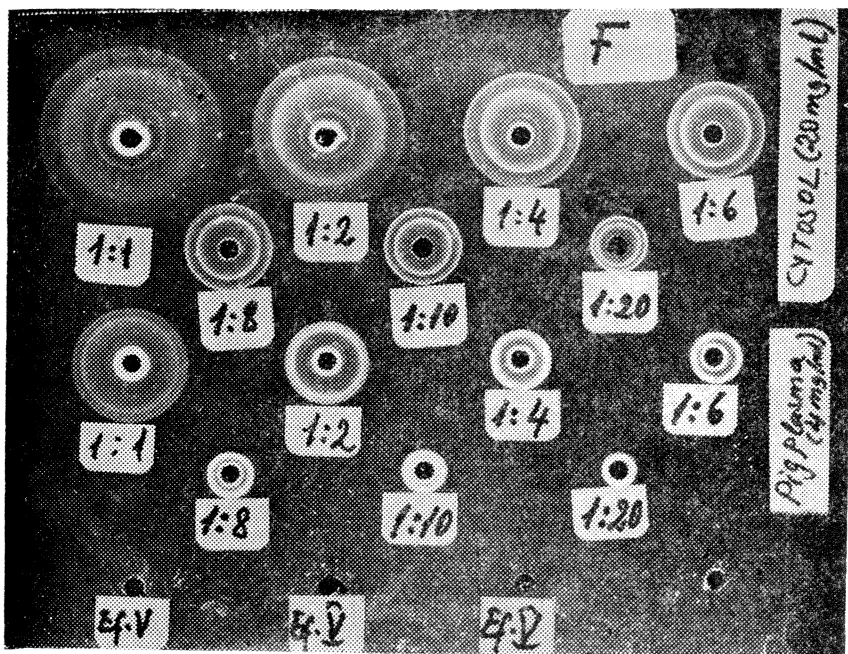
Desorption of immunoadsorbent: For the examination of desorption of immunoadsorbent a small column (6.9 ml) was packed and 6.5 ml of cytosol containing 5.3 mg/ml was applied at 4°C with 4.5 ml/hour velocity and desorbed with different desorption methods (6):

- 1: acidic pH + salt (7)
- 2: » + detergent (8)
- 3: » + salt + detergent + polarity reducing agent
- 4: alkali pH + » + » + »
- 5: chaotropic ions + detergent (neutral pH) (9)
- 6: urea + polarity reducing agent + low salt (neutral pH) (10).



Photograph E: Immunolectrophoresis of effluents

- I: Pig serum (100 mg/ml).
- II: Cytosol (100 mg/ml).
- III: Effluent I (100 mg/ml).
- IV: » II (100 mg/ml).
- V: » III (100 mg/ml).
- VI: » IV (100 mg/ml).
- VII: Desorption with 8 M urea + 50 mM ethanolamin pH 7.4 (100 mg/ml).



Photograph F: and G: The single radial immunodiffusion test of effluents or cytosol. The agar gel contained 0.5 ml (Photograph F) and 1 ml (Photograf G) of goat serum against pig serum. The antigen wells were filled with serial dilutions of cytosol (20 mg/ml) or plasma protein (4 mg/ml) for control or effluent V.

For each test a new batch of immunoabsorbent was used.

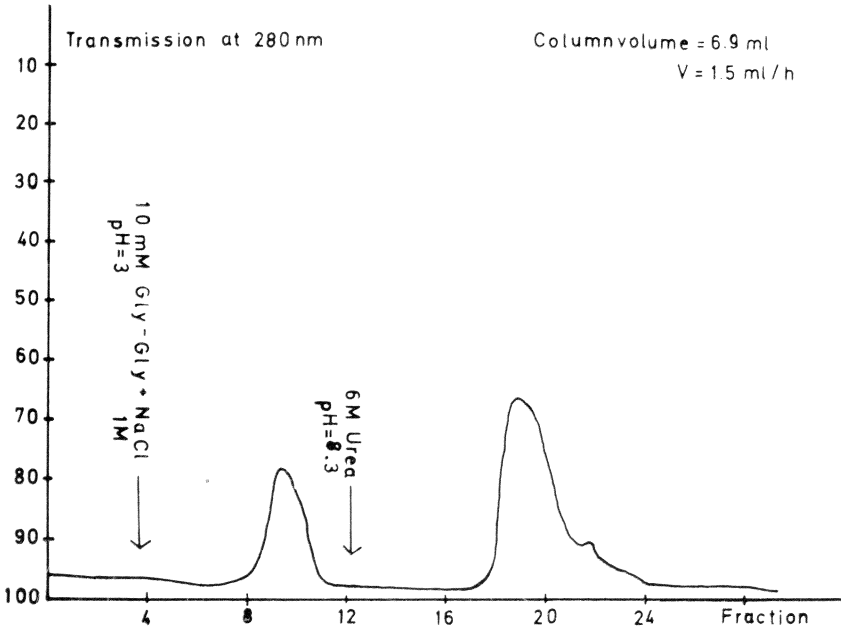


Fig.3: 6.9 ml of immunoabsorbent was packed in a column and 6.5 ml cytosol containing 5.3 mg/ml protein was applied, washed with PBS, 10 mM Gly-gly, 1 M NaCl, pH 3 and 6 M urea pH 8.3.

Table III. The recovery of the desorption of immunoabsorbent with a column volume of each desorbing agent.

Method	recovery (%)
10 mM Na phosphate, 1M NaCl, pH 3	5.8
0.5 M acetic acid, 1% triton X-100, pH 2.5	11.2
0.1 M Na acetate, 2M NaCl, 0.2% X-100, 5% N,N dimethyl formamide	30.4
3 M NaSCN, 0.1% triton X-100 in PBS	58.5
8 M urea, 50 mM ethanolamin, in PBS	40.2

The recovery for desorption of immunoabsorbent with acidic pH + salt is very little. If detergent is used instead the recovery is higher but not optimal. Chaotropions + detergent gives high recovery but it inactivates the immunoabsorbent. High concentra-

tion of urea with low salt and polarity-reducing agent was found to give good recovery which caused no inactivation of the immuno-adsorbent (photograph E).

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