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IDENTIFICATION OF WATER-INSOLUBLE MEMBRANE PROTEINS BY IMMUNOELECTROPHORESIS IN A SOLUBILIZING UREA–TRITON SOLVENT

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SUMMARY

A procedure was developed which renders possible the identification of water-insoluble membrane proteins by immunoelectrophoresis in a solubilizing solvent. Purified erythrocyte membranes from the pig were extracted with butanol, and the protein components were solubilized in a Tris–HCl buffer containing urea and Triton X-100 at reduced concentrations throughout the procedure. After removal of 12% insoluble protein by centrifugation, 50% of the remaining proteins appeared to be monomers and 50% polymers, *i.e.* tetramers. Immunoelectrophoresis in the above buffer mixture resulted in six precipitation lines. The specificity of the reaction was established. It seems that the multiple-site interactions of antigen and antibody have sufficient energy to allow some reduction by addition of the solubilizing agents, urea and Triton X-100, without losing specificity.

INTRODUCTION

Immunoelectrophoresis is widely used for identification and homogeneity control of soluble proteins according to the high specificity of immunological reactions. An extention of this method to water-insoluble membrane proteins is hampered because solubilizing media with ionic detergents, or high molarities of urea and phenol, respectively, denature proteins and interfere not only with the intermolecular interactions of membrane proteins but also with the immunoprecipitation reaction. We have now found a suitable buffer mixture, which achieves both the prevention from reaggregation of solubilized lipid-depleted membrane proteins and the preservation of immunoprecipitation to a high degree. The first results of our studies on erythrocyte membranes were recently reported[1].

MATERIALS AND METHODS

Preparation of the erythrocyte membranes

4 vol. of fresh pig blood were mixed with 1 vol. of 3.8% sodium citrate. The erythrocytes were washed and lysed as described by DODGE *et al.*[2]. Erythrocyte membranes were collected from the hemolysate by centrifugation with the Szent-Györgyi and Blum continuous flow system of the Servall centrifuge (35000 × *g*, flow rate 45 ml/min). The remaining hemoglobin and other water-soluble proteins were removed

by resuspending and washing the pellet 10 times in about 3 vol. of 7 mM phosphate buffer (pH 7.4) and recentrifuging at 35000 × *g* for 20 min. The final pellet was resuspended in the above buffer and stored at −20°. Plasma, serum and hemoglobin from the pig for absorption of immune sera were also kept at −20°.

Immunization procedure and absorption of antiserum

Rabbits of 2 kg weight were immunized against the purified erythrocyte membranes by three subcutaneous injections in the legs at intervals of 2 weeks. Each injection mixture contained 0.3 ml of complete Freund's adjuvant (Travenol International) and lyophilized membranes of 20 mg protein content. 1 week after the last injection, the rabbits were bled by cardiac puncture. The antisera were stored at −20°.

As a control for antibodies against possible contaminants in the erythrocyte membrane preparation, small portions of antisera were absorbed[3] with plasma, serum and hemoglobin from the pig at 37° for 2 h.

Solubilization of the membrane protein

The membrane proteins were solubilized by a two-step procedure. All operations except lyophilization were performed at 4°. At first a butanol extraction for removing the membrane lipids was performed. Membranes of 20 mg protein content, suspended in the 7 mM phosphate buffer, were dialyzed against distilled water and lyophilized. After suspension in 2 ml of *n*-butanol, the material was poured into a dialysis tubing (Kalle, Wiesbaden) of 25–80 Å pore size which had been successively soaked in water, ethanol and butanol, each solvent for 1 day. After removal of air bubbles, the tubing was closed with clamps and hung in *n*-butanol by two cords. Dialysis was performed against 200 ml of *n*-butanol with magnetic stirring and shaking for 24 h. The butanol was then removed by lyophilization at about −5°. In spite of its melting-point at −90°, the butanol did not flash away in the vacuum, provided its layer was thin.

To overcome the strong protein–protein interactions in the second step of solubilization, lipid-depleted membrane residues were suspended by means of a rotation mixer (Eppendorf, Hamburg) in 1 ml of 0.1 M Tris–HCl buffer (pH 8.7) containing 2 M urea and 2 % Triton X-100. After dialysis against the same solvent overnight for removal of possible traces of butanol, the proteins appeared as a clear solution. Insoluble components were spun down in the swinging bucket rotor SW-25 of the Spinco centrifuge (Beckman Instruments) at 75000 × *g* for 15 h. The supernatant was subjected to immunological examination.

Gel filtration for estimation of molecular weights of the solubilized proteins

The special solvent for gel filtration contained 0.1 M Tris–HCl buffer (pH 8.7), 1.5 M urea and 2 % Brij 35 (polyethylene glycol monolauryl ether from Serva, Heidelberg). Brij 35 was used instead of Triton X-100 to obtain a low background absorbance for monitoring the effluent at 280 nm. Sephadex G-200 superfine (Pharmacia, Uppsala) was equilibrated in this solvent for 3 days and degassed. The bed volume was 60 ml in a column of 1.2 cm diameter. A peristaltic pump (LKB, Stockholm) provided a constant flow rate of 1.5 ml per h. The temperature was 10° throughout the procedure. The following calibrating proteins (collection MS-1 from Serva, Heidelberg) were used, each 1 mg per 0.2 ml sample volume: cytochrome *c*, chymotrypsinogen A, ovalbumin,

bovine serum albumin, human γ-globulin and dextran blue. Erythrocyte membrane proteins were applied in samples of 3.4 mg per 0.2 ml of the above solvent.

Immunoelectrophoresis and immunodiffusion

After they had been heated to 100°, 3 ml of 2 % agarose (Serva, Heidelberg) in 0.1 M Tris–HCl buffer (pH 8.7) containing 1.5 M urea and 2 % Triton X-100, were plated on a microscope slide. The gel punch for immunoelectrophoresis (Gelman Instruments, Ann Arbor) was modified in such a way that the wells for the antigens had a diameter of 3 mm. The distance between the rims of the antigen well and antiserum trench was then 2.5 mm. 8 μl of a 1.7 % membrane protein solution were applied. Electrophoresis under water-cooling (4°) was run at 275 V and 3 mA per slide for 1 h. Cytochrome *c* was used as a visible marker. After electrophoresis, 100 μl of antiserum were added which diffused in a moist chamber at room temperature for 48 h. The gels were then washed with a 0.9 % NaCl solution under magnetic stirring for 2–3 days. This saline solvent was changed several times to remove the detergent completely. The gels were dried under filter paper and staining was performed with 0.5 % amido black in methanol–acetic acid (9:1, v/v) for 5 min. The destaining of the gels with methanol–acetic acid (9:1, v/v) usually employed was essentially improved by application of 50 % (v/v) 2-chloroethanol for 1 min between three changes of the methanol–acetic acid.

When the gels were prepared for immunodiffusion, the concentrations of urea and Triton X-100 were lowered to 1 M and 1 %, respectively. Likewise, the butanol-extracted membrane proteins were dissolved in 0.1 M Tris–HCl buffer containing the reduced concentrations of 1 M urea and 1 % Triton X-100. Without centrifugation, samples of 8 μl were diffused for 48 h against the antiserum. Further treatment was identical with that described for immunoelectrophoresis.

Additional methods

Extraction of glycolipids from the erythrocyte membranes was carried out by means of 83 % (v/v) ethanol according to KOŚCIELAK[4]. Mucoids were extracted by a phenol–water distribution as described by KLENK AND UHLENBRUCK[5]. Proteins were determined according to LOWRY *et al.*[6]. After complete removal of the phosphate buffer, the phosphorus content of the membranes was assayed by the method of NORTON AND AUTILIO[7]. Erythrocyte membrane-bound acetylcholinesterase was identified by staining on the agarose gel by the acetylthiocholine–2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride method[8].

RESULTS

Erythrocyte membranes from the pig were obtained in a highly purified state. Contamination with hemoglobin amounted to less than 2 % of the membrane proteins, representing about 0.01 % of the original hemoglobin content of the erythrocytes[9]. The yield was 945 mg membrane protein per l of blood.

A lipid extraction was performed as a first step for obtaining solubilized membrane proteins. When chloroform–methanol (2:1, v/v) or phenol–acetic acid–water (2:1:1, w/v/v) were used for lipid extraction, no precipitation lines of membrane proteins appeared on immunoelectrophoresis with the urea–Triton solvent. After extraction with 2-chloroethanol–water (9:1, v/v) two faint lines were obtained. In

contrast, extraction with butanol followed by immunoelectrophoresis resulted in six precipitation lines, indicating the mild conditions of the procedure. By the butanol extraction, 93.3 ± 1.7 % (S.D., $n=6$) of the membrane-bound phosphorus was removed as phospholipid (Table I).

The choice of solvents for the second step of solubilization of the lipid-depleted membrane proteins is hampered by the requirement that the solvents must not interfere with the immunity reaction. As extreme pH values, high ionic strengths and ionic detergents are known to prevent the immunoprecipitation (see DISCUSSION), a solvent with nonionic compounds was applied. The 0.1 M Tris–HCl buffer (pH 8.7), containing 2 M urea and 2 % Triton X-100, solubilized the lipid-depleted membrane proteins to such an extent that only 12 % of the total protein was in the pellet upon centrifugation at 75000 × *g* for 15 h. Addition of 1 mM disodium EDTA for chelation of Ca^{2+} had no substantial effect on the solubilization (Table II). A buffer with the high pH of 8.7 was chosen because isoelectric points of erythrocyte membrane proteins of different species were reported to be between pH 3.7 and 4.8 (refs. 10, 11).

To determine whether the proteins in the supernatant were in the monomeric state or polymeric aggregates, their molecular weights were estimated by gel filtration

TABLE I

CONTENT OF PHOSPHORUS OF ERYTHROCYTE MEMBRANES BEFORE AND AFTER EXTRACTION WITH BUTANOL

Values are given with the standard error of the mean and the number of experiments in parentheses.

	Dry wt. (*mg*)	*Phosphorus* (*μg*)	*Phosphorus* (%)
Erythrocyte membranes	1.00	18.46 ± 0.83	100 ± 4.5 (6)
Membrane residues after butanol extraction	0.52 ± 0.02	1.23 ± 0.31	6.7 ± 1.7 (6)

TABLE II

SOLUBILITY OF LIPID-DEPLETED ERYTHROCYTE MEMBRANE PROTEINS UNDER VARIOUS CONDITIONS IN 0.1 M TRIS–HCl BUFFER (pH 8.7) CONTAINING 2 M UREA AND 2 % TRITON X-100

Values are given with the standard error of the mean and the number of experiments in parentheses. Reaction volume 1 ml.

Time of centrifugation at 75000 × g	*Solvent additions*	*Protein content of*		
		Supernatant (*mg*)	*Pellet* (*mg*)	*Pellet* (% *of total protein*)
80 min	—	19.4 ± 0.6	0.4 ± 0.3	2.1 ± 1.3 (3)
	With 1 mM disodium EDTA	19.3 ± 0.3	0.3 ± 0.1	1.7 ± 0.6 (3)
15 h	—	17.4 ± 1.2	2.4 ± 0.9	12.0 ± 4.4 (5)
	With 1 mM disodium EDTA	17.8 ± 1.0	2.0 ± 0.8	10.2 ± 4.1 (5)
	2 % Brij 35 instead of Triton X-100	17.8	1.8	9.3 (1)

on Sephadex G-200 superfine. The solvent was essentially the same as used for the immunoelectrophoresis. It contained 0.1 M Tris–HCl buffer (pH 8.7), 1.5 M urea but 2 % Brij instead of 2 % Triton X-100. This solvent diminished the nominal exclusion limit of the Sephadex gel from the molecular weight of 800000 to values slightly above that of γ-globulin (160000). As can be seen from Fig. 1, the relationship between ratios of elution volume to void volume and the logarithm of the molecular weight of the calibrating proteins was linear[12] only in the range from molecular weight 12400 (cytochrome *c*) to 67000 (bovine serum albumin). The γ-globulin was eluted next to the void volume being partially excluded from the gel. More than 95 % of the erythrocyte membrane proteins were recovered in two peaks. Each peak represented about 50 % of the applied protein. Their molecular weights were scattered around 64000 and 180000. However, since the shapes and specific volumes of the membrane proteins are unknown, the estimation of their molecular weights by comparison with the spherical calibrating proteins is only approximate.

Fig. 2a shows the results of an immunodiffusion in 0.1 M Tris–HCl buffer (pH 8.7) without additions. Erythrocyte membranes, hemoglobin, serum and plasma from the pig diffused against the antiserum from the rabbit. Regardless of whether the antiserum was absorbed with the latter three protein samples or not, only the erythrocyte membranes formed two precipitation lines, thereby demonstrating the specificity of the antiserum.

In contrast, Fig. 2b represents an immunodiffusion in Tris–HCl buffer (pH 8.7) containing 1 M urea and 1 % Triton X-100. The lipid-extracted membrane proteins formed four lines, although they did not appear to be monomeric ones prior to precipitation. This was indicated by the insufficient separation of the precipitation lines, when immunoelectrophoresis was carried out at those urea–Triton concentrations. In both modifications of diffusion (Figs. 2a and 2b), the major portion of the proteins remained insoluble in the antigen well. But an increase of the urea and Triton concentrations was impossible because they interfere with the immunoprecipitation.

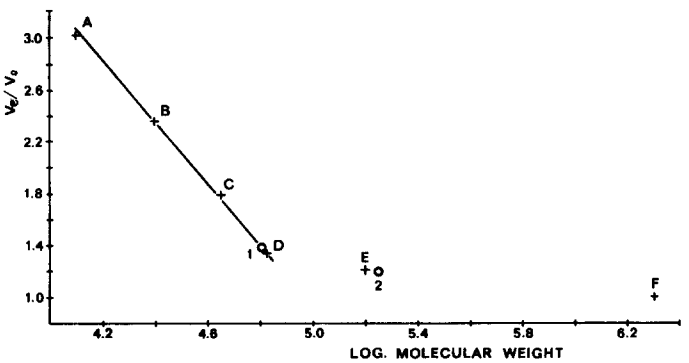

Fig. 1. Estimation of molecular weights of the erythrocyte membrane proteins by gel filtration on Sephadex G-200 superfine, equilibrated with 0.1 M Tris–HCl buffer (pH 8.7) containing 1.5 M urea and 2 % BRIJ 35. The calibrating line of the ratios of elution volume (V_e) to void volume (V_0) *vs.* logarithm of the molecular weight of the standard proteins (A–F) was plotted from the mean value of three experiments. Standard proteins: (A) cytochrome *c*, mol. wt. 12400; (B) chymotrypsinogen A, mol. wt. 25000; (C) ovalbumin, mol. wt. 45000; (D) bovine serum albumin, mol. wt. 67000; (E) human γ-globulin, mol. wt. 160000; (F) dextran blue, mol. wt. 2000000. The erythrocyte membrane proteins (circles 1 and 2) eluted as two peaks of molecular weights around 64000 and in the range of 180000, each containing about 50 % of the total protein.

A typical example of an immunoelectrophoresis in 0.1 M Tris–HCl buffer (pH 8.7) containing 1.5 M urea and 2 % Triton X-100 is illustrated in Fig. 3a. A maximum of six precipitation lines appeared, if the proteins were first dissolved in the buffer containing 2 M urea and 2 % Triton X-100 as described. In order to render the immunoprecipitation possible, the reduction from 2 M urea in the sample to 1.5 M urea in the gel was necessary. The addition of the 100 μl urea-free antiserum after electrophoresis as well as the uptake of humidity in the moist chamber resulted in a further, necessary diminution of the final urea–Triton concentration. To check whether this decrease of the urea and Triton concentration could result in an unspecific precipitation of the membrane proteins, and to exclude the possibility of cross-reactions in this solvent, an immunoelectrophoresis was carried out with diffusion against an anti-human serum from the rabbit. The result is documented in Fig. 3b. Aggregated proteins were only found near the antigen well, as in Fig. 3a. These aggregates may represent the protein fraction that was eluted on Sephadex gel filtration in the molecular range as high as 180000. It seemed to have the tendency to aggregate on further dilution of the solvent. Therefore it was not removed by the subsequent washing procedure. In contrast to the immunodiffusions, no insoluble protein remained in the antigen well.

To exclude the possibility that the precipitated antigens may represent the well-known blood group substances such as glycosphingolipids[4] and mucoids[5], the corresponding extraction procedures were performed on butanol-extracted membrane residues. No precipitation lines appeared on immunoelectrophoretic examination, even when 5 times concentrated extracts were studied. Thus the protein nature of the precipitated antigen can be assumed.

Identification of some electrophoretically separated membrane proteins is possible not only by their immunological but also by their catalytic reactions. Fig. 4 represents the reaction products of acetylcholinesterase of the erythrocyte membrane

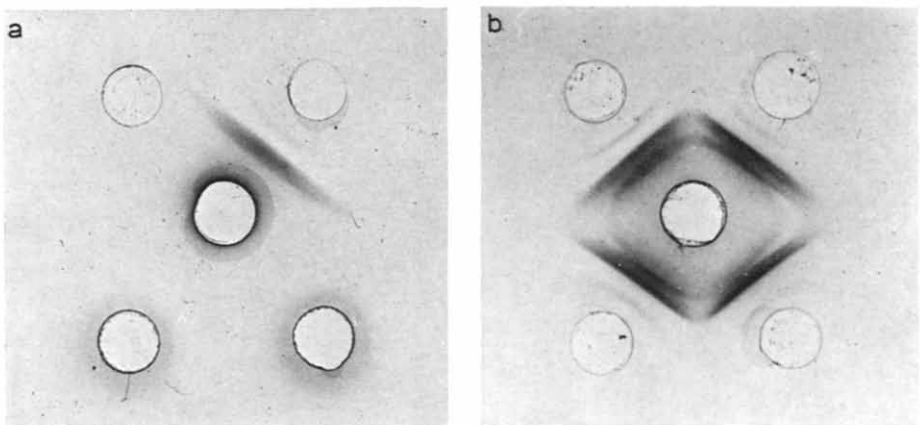

Fig. 2. a. Immunodiffusion in 0.1 M Tris–HCl buffer (pH 8.7) without urea or Triton. Hemoglobin (left side above), serum (left side below), plasma (right side below) and erythrocyte membranes (right side above) from the pig; membrane antiserum from the rabbit (central well). Only the erythrocyte membranes form two precipitation lines. The ring-shaped shadow around the central well results from peeling off the gel. b. Immunodiffusion of lipid-extracted erythrocyte membrane proteins in 0.1 M Tris–HCl buffer (pH 8.7) containing 1 M urea and 1 % Triton X-100. The membrane proteins form four precipitation lines.

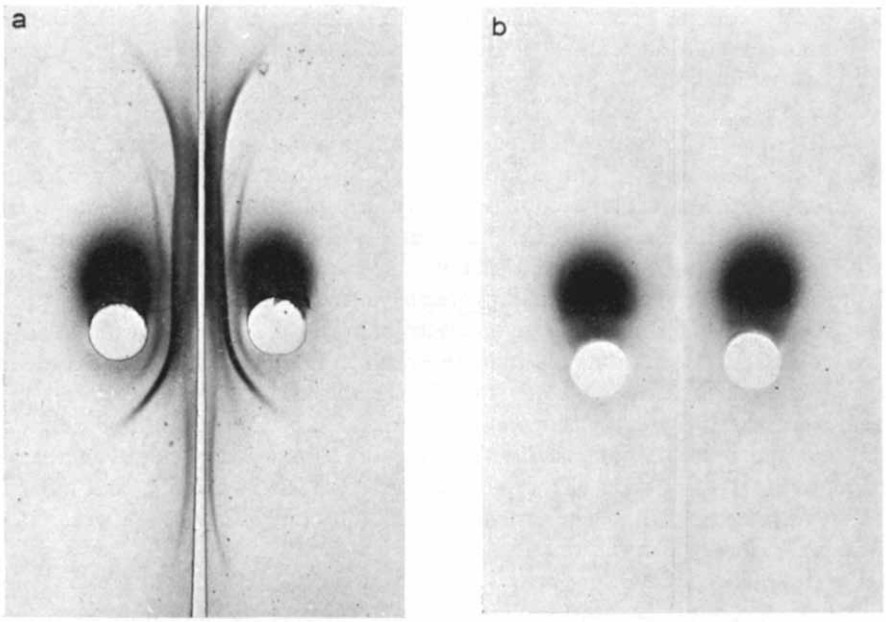

Fig. 3. a. Immunoelectrophoresis of erythrocyte membrane proteins in 0.1 M Tris–HCl buffer (pH 8.7) containing 1.5 M urea and 2 % Triton X-100. Six precipitation lines are to be seen as well as unspecifically aggregated proteins near the antigen well. b. Control experiment under the same conditions but with the membrane antiserum replaced by anti-human serum. Only unspecifically aggregated proteins appear near antigen well.

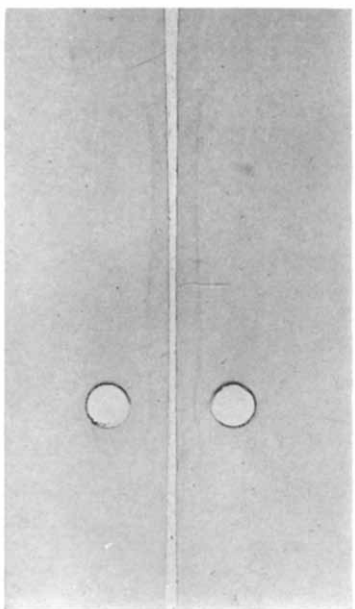

Fig. 4. Specific staining of the acetylcholinesterase after immunoelectrophoresis under the conditions described for Fig. 3a. The enzyme is localized in the region of the main precipitation line of Fig. 3a.

upon immunoelectrophoresis. The enzyme is located in the region of the main precipitation line which is usually seen on staining with amido black. The double-arched line points to the existence of several forms of the enzyme with different electrophoretic mobilities, perhaps due to partial dissociation into its subunits[13]. The specificity of the staining by the enzyme action was assured by a control experiment in the presence of 10 μM physostigmine.

DISCUSSION

The basic problem for the identification of membrane proteins by immunoelectrophoresis is the necessity of solubilizing them without denaturation. Because the two-phase method of butanol extraction described by MADDY[10] for erythrocyte membranes results in loss of proteins at the interface and does not allow a longer storage of the ghosts in the frozen state, we employed the single-phase procedure described. Reducing the denaturation by reorientation of the hydrophilic and hydrophobic side-chains by interfacial forces, this procedure avoids loss of proteins. The efficiency with regard to removal of lipids is the same. In contrast with the various butanol methods developed by MORTON[14] for solubilization of membrane-bound enzymes, washing with acetone was avoided, and membrane preparations were treated with butanol solvent after removal of inorganic salts, as introduced by MADDY to minimize interfacial forces.

Because, after extraction with butanol, protein molecular weights of 300000 (ref. 10) and 4.6–32-S components[10,11,15] were obtained, we reduced the strong protein–protein interactions by the urea–Triton X-100 solvent. Removal of the insoluble components required centrifugation for 15 h at 75000 $\times$ g, a rather long time when compared with the usually reported criterion of solubility, 1 h at 100000 $\times$ g. Under the latter conditions, 98 % of the proteins remained in the supernatant, but under the former only 88 %. On gel filtration, the apparent molecular weights of the two protein fractions of the supernatant, each comprising 50%, ranged around 64000 and 180000. These values are only approximate because the shapes and specific volumes of the membrane proteins are unknown. It has been reported that carbohydrates show, relative to their sizes, a behavior different from that of proteins on Sephadex columns by lowering the exclusion limit[12]. The carbohydrate moiety accounts for about 9% of the erythrocyte membrane proteins by weight[15] and may therefore result in too high molecular weight findings. On the other hand, the weights as determined are to some extent in agreement with previously reported values obtained by polyacrylamide-gel gradient electrophoresis with phenol–formic acid–water 14:3:3 (w/v/v) solvent[9]: 92% of the erythrocyte membrane proteins of the pig had been found in the range between 26000 and 65000. For 8% with an estimated weight in the order of 180000, the monomeric state could not be assured. In the urea–Triton X-100 mixture used for the immunoelectrophoresis, 50% of the proteins may be present as monomers and subunits, and 50% could represent tetramers, for instance.

Solubilization of membrane proteins and precipitation by immunological reaction at the same time might be considered to be impossible because the membrane protein–solvent interactions must be stronger than the interactions between the membrane proteins. Therefore, the former will also compete with the specific antigen–

antibody interactions and with the interactions between the formed, soluble, immune complexes that are necessary for precipitation. However, our results indicate that a simultaneous solubilization and precipitation is possible.

In essential agreement with reported values[16], we found the precipitation reaction to an increased degree inhibited on either side of the pH range 5.7–8.7 and also by the presence of 0.1 % sodium dodecyl sulfate. This may be due to electrostatic repulsion among protein molecules, as assumed for the failure of precipitation without destruction of binding sites by acetylation of amino groups, which results in a relative increase of negative charges[17]. For this reason, we employed a buffer of pH 8.7 with nonionic solubilizing additions. Another approach was made by CORRY AND STONE[18], who found one to two precipitation lines with erythrocyte stroma in a physiological buffer after previous solubilization in 1 % sodium dodecyl sulfate and its subsequent removal.

To guarantee the necessary specificity and selectivity, the antigen–antibody interactions must represent "multiple-site" interactions with a predetermined spatial arrangement[19]. Below pH 4.5 and above pH 9, increasing conformational changes of γ-globulin molecules were observed[20] in agreement with the finding that soluble immune complexes in the inhibition zone dissociated below pH 4.6 (ref. 21). The reason for the decrease of immunoprecipitation with increasing salt concentration was explained by changes in combination of antibody and antigen, but not by increased solubility of the complexes[22]. In contrast to the strong influence of pH and ionic strength, we found that 2 % Triton X-100 had no essential effect on the immunological reaction. It was therefore used for solubilization of the membrane proteins, because their association may be based to a high degree on hydrophobic interactions. This is indicated by the fact that most of the known membrane proteins consist, in relatively high degree, of amino acids with apolar side-chains. The solubilization was essentially improved by the addition of urea, whose solubilizing efficiency does not only depend upon its prototropic properties, but also on its ability to weaken hydrophobic interactions[23].

FREEDMAN *et al.*[24] described the dissolution of specific precipitates of different proteins in 8 M urea without dissociation of the antigen–antibody complexes. On the other hand, NISONOFF AND PRESSMAN[25] reported that under the condition of equilibrium dialysis the binding power of rabbit antihapten antibody is reversibly diminished with 2 M urea by 60 % of the total decrease observed in 8 M urea. At a concentration of 2 M urea the conformation of γ-globulin is not markedly changed[20, 25, 26]. Therefore, these observations were explained by a direct competition between urea and the hapten for the binding site of the antibody[25]. The hapten, *p*-iodobenzoate, is not as capable of multiple-site interactions with antibodies as the much larger protein antigens. Thus the influence of urea will not be so effective on the protein antigen–antibody binding. Furthermore, it will be of importance whether single molecules as in the experiments of NISONOFF, or formed immune complexes as in the experiments of FREEDMAN, are exposed to urea. In our experiments, a gradual diminution of urea occurred in the range between 2 and 1 M. A marked increase of precipitating activity in this range was established.

It seems that the multiple-site interactions of antigen and antibody have sufficient energy to allow some reduction without loss of specificity. Therefore, urea and Triton X-100 can be employed for prevention of reaggregation of solubilized mem-

brane proteins at the same time as specific immunoprecipitation occurs. Thus the immunoelectrophoretic method described is suitable for the identification of membrane antigens of different origins.

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