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Specific antiserum staining of two-dimensional electrophoretic patterns of human plasma proteins immobilized on nitrocellulose

Human plasma proteins separated by high-resolution two-dimensional electrophoresis have been electrophoretically transferred to sheets of nitrocellulose using a modification of the method of Towbin, Staehelin, and Gordon [8]. Although the proteins have been denatured in sodium dodecyl sulfate and separated into subunits, the nitrocellulose-bound molecules still react with appropriate specific antisera even after storage of the transfer in air at room temperature for 5 months. Of 25 proteins whose location in the pattern had been previously determined, 24 are specifically revealed on transfers of whole plasma patterns by appropriate antiserum. In addition, 6 previously unidentified proteins (prothrombin, C1s, C4γ, C1s inhibitor, Ig J-chain, and a_1AP glycoprotein) have been identified in the pattern for the first time using the transfer technique. It therefore seems likely that a large majority of proteins (> 96 % in this study) retain sufficient conformation throughout the analytical procedure (or can regain it easily afterwards) to be recognized immunologically. The transfer technique thus constitutes a generally useful immunological "third-dimension" in the high-resolution separation of proteins. Of three monoclonal antibodies similarly tested, none could detect antigen transferred to nitrocellulose from a two-dimensional gel, while each bound specifically to the appropriate antigen absorbed in native form to the nitrocellulose.

1 Introduction

High resolution two-dimensional electrophoretic separations make it possible for the first time to consider cataloging and studying the large numbers (thousands) of proteins that constitute the working parts of cells. One possible drawback of this technique is that the amounts of protein separated are generally quite small, and appear in a "denatured" condition owing to their exposure to high concentrations of urea followed by sodium dodecyl sulfate (SDS). Although enzymatic and immunological activities have occasionally been recovered in proteins treated with such denaturants, it has been generally supposed that the majority of proteins would thereby lose most or all of their interesting biological properties. The retention of some of these properties would allow the extension of the two-dimensional electrophoretic system into a third "specificity" dimension. Two-dimensional electrophoresis could thus become an intermediate, rather than the final analytical stage.

In 1976 Burridge reported that some antibodies could recognize specific proteins among those banded on a one-dimensional SDS gel [1], thus extending a technique developed earlier for "staining" glycoproteins in gels with iodinated lectins [2, 3]. Since Burridge's technique involved the diffusion of antibody molecules into and out of the polyacrylamide matrix of the SDS-gel, it was slow and progressively more difficult for lower porosity gels. These difficulties were removed by the introduction of methods for transferring the pattern of separated proteins out of the acrylamide gels and onto thin and therefore more accessible substrates in imitation of the highly successful "Southern" blot transfers used in nucleic acid research [4]. Useful substrates include diazobenzyloxymethyl (DBM) paper [5, 6, 7], m-amino styrene substituted low-porosity acrylamide (N. L. Anderson and S. L. Nance, unpublished) and nitrocellulose [8]. The nitrocellulose method is particularly appealing because of the absence of any chemical steps; here the proteins are adsorbed purely by non-covalent forces.

We have several aims in adapting the protein transfer technology to the analysis of high-resolution two-dimensional electrophoretic patterns. The first is the easy identification on gel patterns of proteins against which antibodies are available. Although immunoprecipitates can usually be prepared and subsequently analyzed in two-dimensions to afford such identifications [9], there are many circumstances (such as the immunoprecipitation of immunoglobulin chains or complement components from serum) in which complicating factors arise, and a more direct method is desirable. A second aim is the characterization of natural and pathological autoantibodies, and a third is the preparation of small amounts of pure antibody against cellular proteins which have themselves never been purified except by two-dimensional electrophoresis. In the latter instance an antiserum containing antibodies against many proteins may be applied to a nitrocellulose transfer, and the antibodies which selectively attach to one spot may be eluted. In order to undertake such a program, it is desirable to know what proportion of proteins retain antigenic sites after two-dimensional electrophoresis sufficient for recognition by conventional antisera or monoclonal antibodies. The answer determines whether the method can be used generally or only in a restricted number of instances.

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Abbreviations: SDS: Sodium dodecyl sulfate; BSA: Bovine serum albumin; HSA: Human serum albumin; Con A: Concanaualin A

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2 Materials and methods

2.1 Two-dimensional electrophoresis

Human plasma proteins were resolved in two dimensions using the ISO-DALT system previously described [10, 11]. Briefly, this involves dilution of plasma in 2 % SDS, 50 mM cyclohexylaminoethane sulfonic acid (CHES buffer) pH 9.5, 10 % glycerol 1 % dithiothreitol, and isoelectric focusing in 9 M urea/2 % NP-40 followed by SDS electrophoresis. The procedure is a "denaturing" one in that protein subunits are dissociated and their tertiary structure loosened or destroyed in both dimensions.

2.2 Transfer of two-dimensional patterns to nitrocellulose

Proteins were transferred from the two-dimensional gel onto nitrocellulose (Schleicher and Schuell) by electrophoresis perpendicular to the plane of the two-dimensional slab. A modified insert for the DALT® tank [11] was constructed to allow transfer of six 6″ × 6″ gels per tank (Fig. 1). During transfer the gel is pressed against a nitrocellulose sheet of the same size between sheets of foam rubber on either side; these are pressed together by pieces of commercially available plastic ("egg crate") lattice material normally used in fluorescent lighting fixtures and held in place by nylon screws. During transfer, the DALT® tank is filled with a buffer consisting of 25 mM Tris base and 193 mM glycine. The process is essentially complete in 4 h at 100 V using the configuration shown in Fig. 1.

2.3 Blocking and staining of transfers

The methods used are modifications of those described by Towbin *et al.* [8]. The nitrocellulose transfers can be stained directly for protein if desired [8] using Amido Black in 45 % ethanol, 10 % acetic acid (Coomassie Brilliant Blue R-250 stains nitrocellulose and cannot be used). For specific antibody staining, the following protocol was followed after the transfer step: (1) Blocking of remaining protein binding sites with 3 % bovine serum albumin (BSA) in Tris buffered saline overnight at 4 °C; (2) Application of desired antibody (dilution in the range 1:50–1:1400) in Tris-saline + 3 % BSA for 30 min at room temperature; (3) Five saline washes; (4) Application of peroxidase-conjugated goat anti-rabbit (or other appropriate) antibody (Miles Laboratories) at 1:1500 dilution in Tris-saline + 3 % BSA for 60 min at room temperature; (5) Five saline washes, (6) Staining briefly with fresh solution of diaminobenzidine/H_2O_2/Tris. Specific mention of steps 3–6 is often omitted in the text for clarity of presentation; "stained with antiserum to x", thus, implies the use of the entire protocol described.

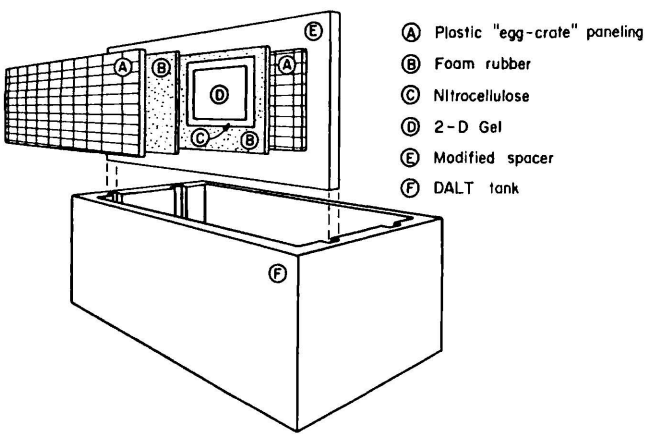

Figure 1. Diagram of DALT tank [11] modified for use as an electrophoretic transfer apparatus. Each of the two removable flap-seal/comb assemblies is replaced by a transfer plate assembly (only one of which is shown in the diagram). The transfer assembly consists of two sheets of plastic "egg crate" lattice material (normally used as a diffuser in fluorescent light fixtures) between which a sandwich of foam rubber-nitrocellulose-gel-foam rubber is compressed by means of nylon screw clamps. A gel is placed on the cathodic side of each nitrocellulose sheet. Each assembly holds three gels, for a total of six gels per tank. Current is passed through the gel-nitrocellulose sandwiches using the platinum electrodes permanently mounted on the long side walls of the DALT tank [11]

2.4 Photography of stained transfers

Transfers were photographed on Kodak Contrast Process Pan film through a blue glass filter with transmission band centered at 424 nm. A Kodak Versamat processor was used to develop the film.

2.5 Monoclonal antibodies

Mouse monoclonal antibodies to human serum albumin and transferrin were made by fusing spleen cells of immunized mice to the myeloma line NS-1, according to the general approach of Kohler and Milstein [12] as described by Pearson *et al.* [13]. Monoclonal antibodies were detected using solid-phase radioimmunoassay [14].

3 Results

The electrophoretic transfer procedure used here results in very efficient removal of protein spots from the original two-dimensional gel slab, with nearly quantitative adsorption to the nitrocellulose sheet (Fig. 2). More than 90 % of the protein is successfully transferred, the remainder consisting primarily of excess albumin, ceruloplasmin, and the low-molecular weight Apo A-II lipoprotein. A striking feature of the transferred pattern is the excellent preservation of the high-resolution pattern present in the original gel; there is no visible spreading (or movement) of spots in the plane of the gel/nitrocellulose sandwich during transfer. Autoradiographs of transfers of labeled protein patterns show higher resolution than autoradiographs of the dried two-dimensional gels themselves (data not shown), an effect probably due to the thinness of a transfer compared to a dried gel, and the associated lack of parallax spreading.

Fig. 3A shows the image produced when a transfer of the whole human plasma two-dimensional pattern is incubated with rabbit antiserum to the Cls piece of complement, and this bound antibody revealed by goat anti-rabbit peroxidase stain. Two small spots are heavily stained, but it is not immediately obvious where these fall in relation to the whole pattern. By staining the transfer a second time, in this case with Concanavalin A (Con A) (to reveal glycoproteins generally) followed by rabbit antibody to Con A, and then

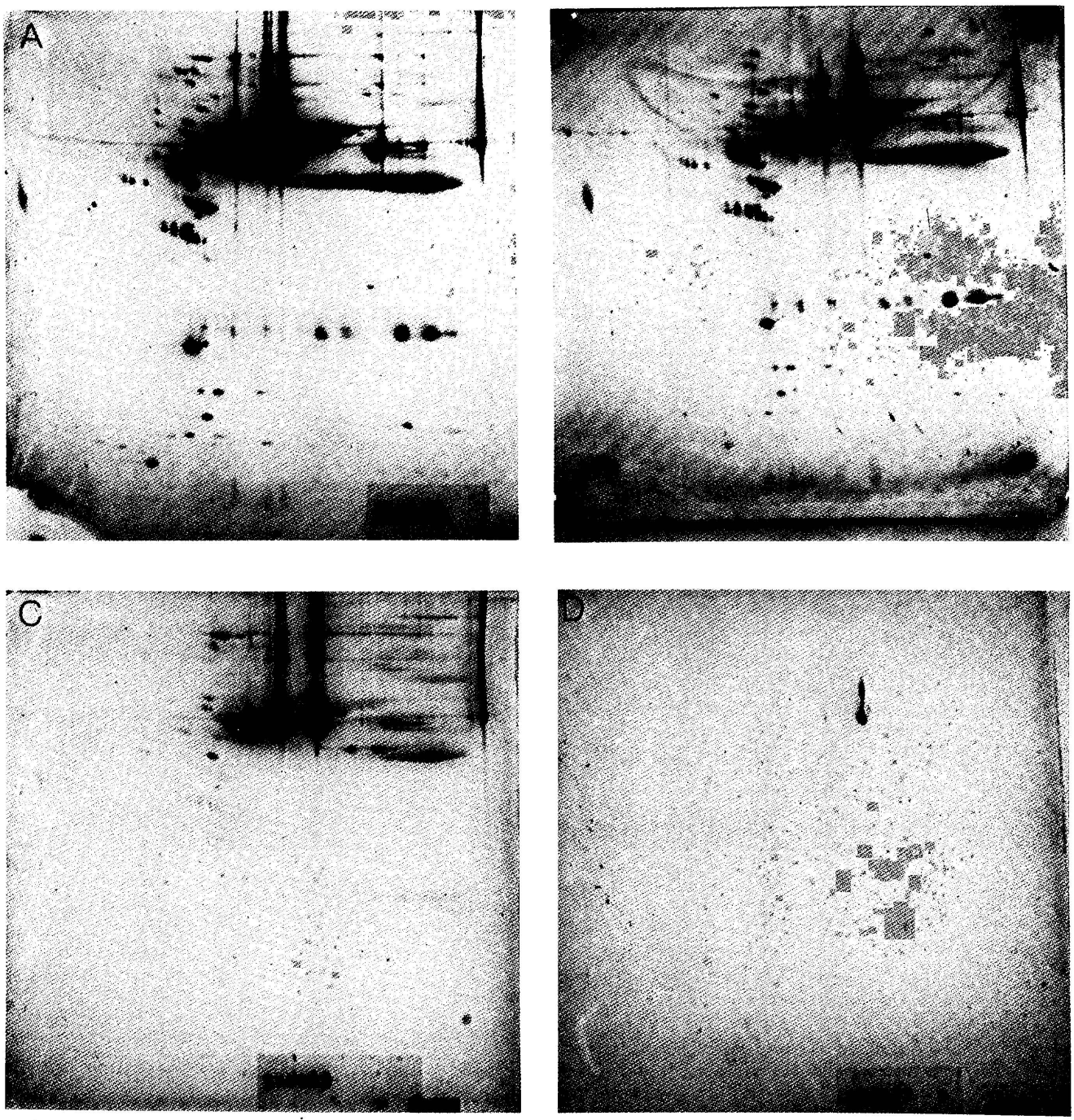

Figure 2. Demonstration of the efficiency of the transfer procedure employed. (A) Coomassie Brilliant Blue R-250 stained two-dimensional gel of human plasma proteins. (B) Amido Black stained nitrocellulose transfer of a plasma protein pattern from another similar gel in the same batch. (C) Gel from which the transfer in (B) was made, stained with Coomassie Brilliant Blue R-250 after the transfer step. Some albumin, ceruloplasmin, Gc-globulin, and fibrinogen β-chain remain, but the vast majority of protein has been transferred. (D) Initially blank gel slab which was placed behind the nitrocellulose sheet B during electrophoretic transfer to retain proteins leaking through the nitrocellulose. Very little protein (some albumin, in this case) fails to bind to the nitrocellulose.

goat anti-rabbit/peroxidase, the location of the Cls spots in the whole pattern is apparent (Figure 3B). Sugar moieties binding Con A are evidently unaffected by a previous staining cycle. On this transfer, as with many of those to follow, some inappropriate spots were stained at low level in addition to the ones constituting the presumed antigen. The "non-specifically" stained spots usually comprise albumin, transferrin, Igμ and γ chains or fibrinogen. Whether this "noise" is due to contamination of the original immunogen, poor absorption, or to genuine cross-reactivity with denaturation-revealed sites on other proteins has not yet been determined. By using affinity purified $F(ab')_2$ portions of antibodies, this problem may perhaps be reduced or eliminated.

A more stringent test of the preservation of antigenic structure on such transfers is shown in Fig. 3C and 3D. Here a plasma protein transfer has been stained with rabbit anti-

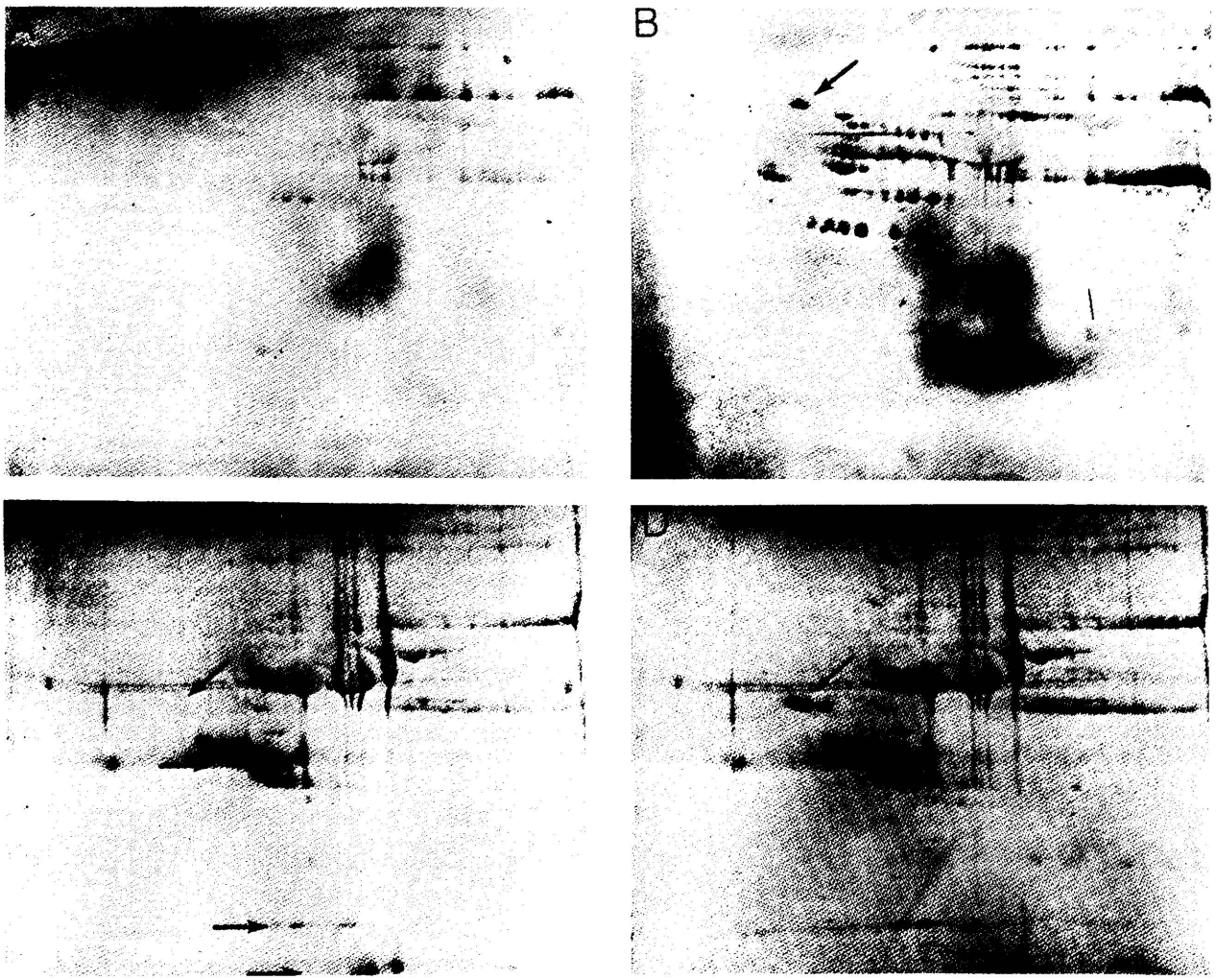

Figure 3. (A) Two-dimensional plasma protein stained with Behring rabbit anti-Cls component followed by Miles Laboratories peroxidase conjugated anti-rabbit Ig antibody and peroxidase stain. Arrow indicates principal stained spot complex. (B) Same transfer as shown in (A), but restained sequentially with Con A, anti-Con A antiserum, and peroxidase conjugated anti-rabbit Ig to reveal major glycoproteins. The relationship of the stained spots in (A) to the whole pattern is apparent in (B) where the arrow again indicates Cls. (C) Two-dimensional plasma protein transfer stained with Behring rabbit anti-haptoglobin (Hp) antiserum. The three horizontal arrows indicate (from top to bottom) the Hp β, α_2, and α_1s chains respectively. (D) Same transfer as in (C) but restained for α_2HS glycoprotein (downward pointing arrow) after 5 months' storage in air at room temperature.

haptoglobin antiserum and goat anti-rabbit/peroxidase (Fig. 3A), dried in air and stored for 5 months at room temperature, and finally restained with anti-α_2HS glycoprotein antiserum. (Fig. 3D). The α_2HS glycoprotein spots are accurately detected by the antiserum despite extended storage under mildly oxidizing conditions (room temperature, air). An important observation is that in the case of haptoglobin, all subunits are specifically stained despite the fact that they are widely separated, and hence unable to interact with each other on the transfer. Evidently the subunits are able to retain or regain some of their immunological reactivity in isolation from one another. Similar results are obtained in the case of fibrinogen (which has three types of chains), confirming this result.

The detection efficiency obtainable with a good antiserum, anti-Ab/ peroxidase and diaminobenzidine is shown in Fig. 4. The peroxidase stain appears to be between 10- and 100-fold more dense than Coomassie Brilliant Blue R-250 in agreement with the detection efficiency estimated by Towbin *et al.* [8]. In this case it allows the detection of a higher molecular weight form of α_2HS glycoprotein present in only trace quantities and invisible by Coomassie Blue staining even on heavily loaded gels. This larger form is probably a precursor of the normal circulating molecule [15]. Fig. 4C-H and Table 1 illustrates the results of staining human plasma nitrocellulose transfers with 19 different specific rabbit antisera capable of recognizing a total of 25 different polypeptides. In every case (except α_1 anti-chymotrypsin) in which the protein's location was known in advance, the antiserum stained the appropiate spot(s). A number of previously unidentified proteins were located in the pattern (including prothrombin [Fig. 4, G and H], Cls, Cls inhibitor, the immunoglobulin J-chain, α_1 AP glycoprotein and part of C4). The positions of these newly identified proteins and those of the previously located proteins are shown on an updated map of the human plasma proteins (Figure 5).

Three mouse monoclonal antibodies to human serum albumin or transferrin did not specifically stain any serum pro-

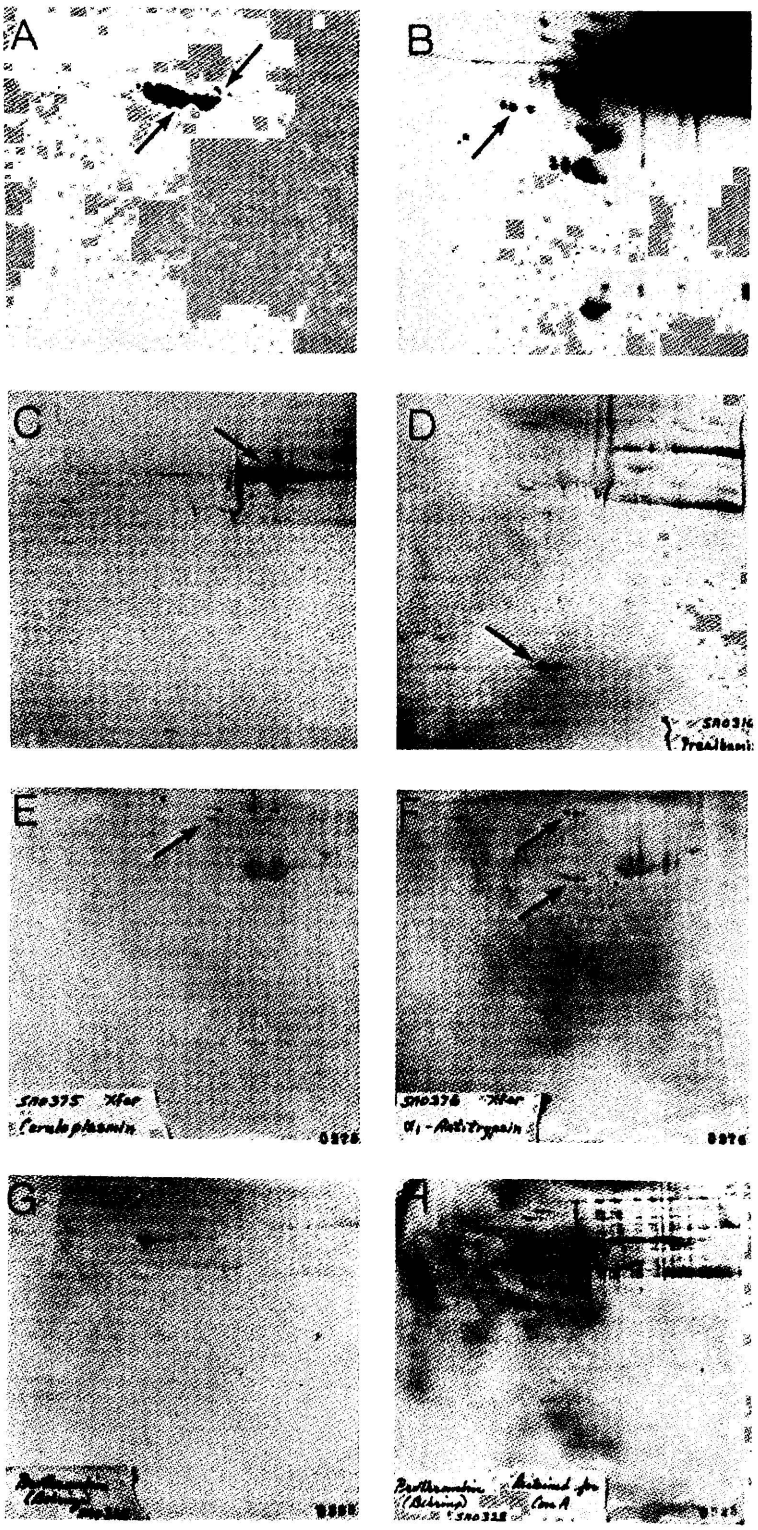

Figure 4. (A) Portion of a transfer showing spots stained by antiserum to α_2HS glycoprotein. Upward arrow indicates the previously recognized [9, 13] major form, while downward arrow indicates an identical protein micro-pattern displaced in pI and at higher SDS-molecular weight. The latter, because of its larger size, probably represents a precursor to the normal circulating species. (B) Portion of a Coomassie Brilliant Blue R-250 stained gel loaded with the same amount of plasma protein as the gel from which the transfer in (A) was made. Arrow indicates α_2HS glycoprotein. (C-F) Plasma protein transfers stained with antisera to transferrin (C), prealbumin (D), ceruloplasmin (E), and α_2 antitrypsin (F; which also detects the dimer form shown by the upper arrow). (G-H) Plasma protein transfer stained for prothrombin (G) and restained with Con A to show unequivocal location of prothrombin (H).

teins transferred from a two-dimensional gel, though they did correctly stain their respective antigen when it was applied to the nitrocellulose as native protein (Fig. 6). Although the number of monclonal reagents tested was small, the results nevertheless contrast markedly with those using rabbit antisera.

In order to obtain some idea of the stability of the protein-nitrocellulose interaction compared to the antigen-antibody interaction involved in the staining steps, albumin labeled with [^{14}C]-iodoacetamide was applied as a spot to small squares of nitrocellulose and subsequently reacted with anti-albumin antiserum. The squares were then washed in various chaotropic agents, stained with goat anti-rabbit/peroxidase to reveal remaining bound antibody, and counted in a liquid scintillation system to determine the quantity of albumin remaining. Exposure to (1) 1.0 M NaCl, (2) various pH's in the range 3.5–9.0, (3) 4 M urea, (4) CO_2-saturated phos-

Table 1. Antibody detection of human plasma proteins on nitrocellulose transfers of two-dimensional electrophoretic patterns

Protein	No. of spot complexes	No. of correct spot complexes detected
Albumin	1	1
Transferrin	1	1
Haptoglobin	3	3
α_1 antitrypsin	1	1
Ceruloplasmin	2	2
Prealbumin	1	1
α_2 macroglobulin	1	1
Plasminogen	2	2
Gc-globulin	1	1
Hemopexin	1	1
Antithrombin III	1	1
α_2HS glycoprotein	1	1
Fibrinogen	3	3
α_1 antichymotrypsin	1	0
IgK	1	1
Igγ	1	1
Igμ	1	1
Igα	1	1
α_1B glycoprotein	1	1
Total	25	24

phate-buffered saline [16], or (5) 3 M ammonium thiocyanate had very little effect in eluting either bound albumin or anti-albumin antibody. However, treatment with 6 M NH_4SCN removed most of the antibody and left virtually all of the albumin adsorbed (Fig. 7). Ammonium thiocyanate elution is therefore a reasonable approach in attempting to prepare small quantities of specific antibodies by elution from small areas of two-dimensional gel transfers.

4 Discussion

Human plasma proteins having a wide range of isoelectric points and molecular weights can be transferred electrophoretically to nitrocellulose sheets where they remain very strongly bound. Individual proteins in the resulting replica pattern can be stained by specific antibodies or lectins. The antigenic sites responsible for specific staining appear to be stable through repeated peroxidase staining procedures separated by many months storage at room temperature. Nitrocellulose transfers thus appear to constitute permanent, reusable (though not yet fully erasable) antigenic "pictures" of the separated constituents of complex protein mixtures,

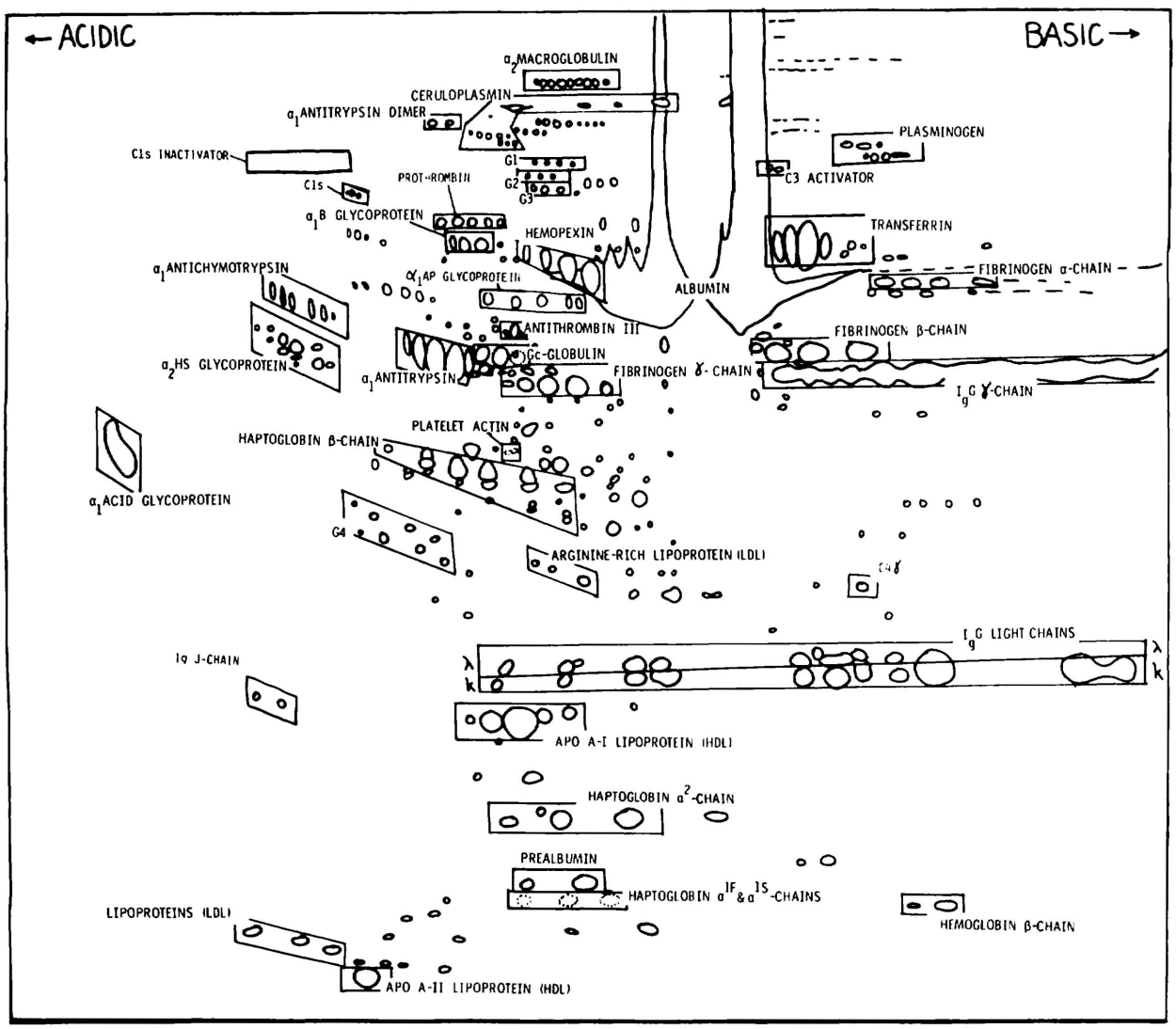

Figure 5. Schematic diagram of the two-dimensional pattern of the human plasma proteins, modified from [9] by the addition of C1s, C1s inhibitor (not usually visible by Coomassie Brilliant Blue R-250 staining), prothrombin, α_1AP glycoprotein, C4γ-subunit, and the immunoglobulin J-chain. The labeling of the three fibrinogen chains has been corrected from [9], where their order was mistakenly inverted.

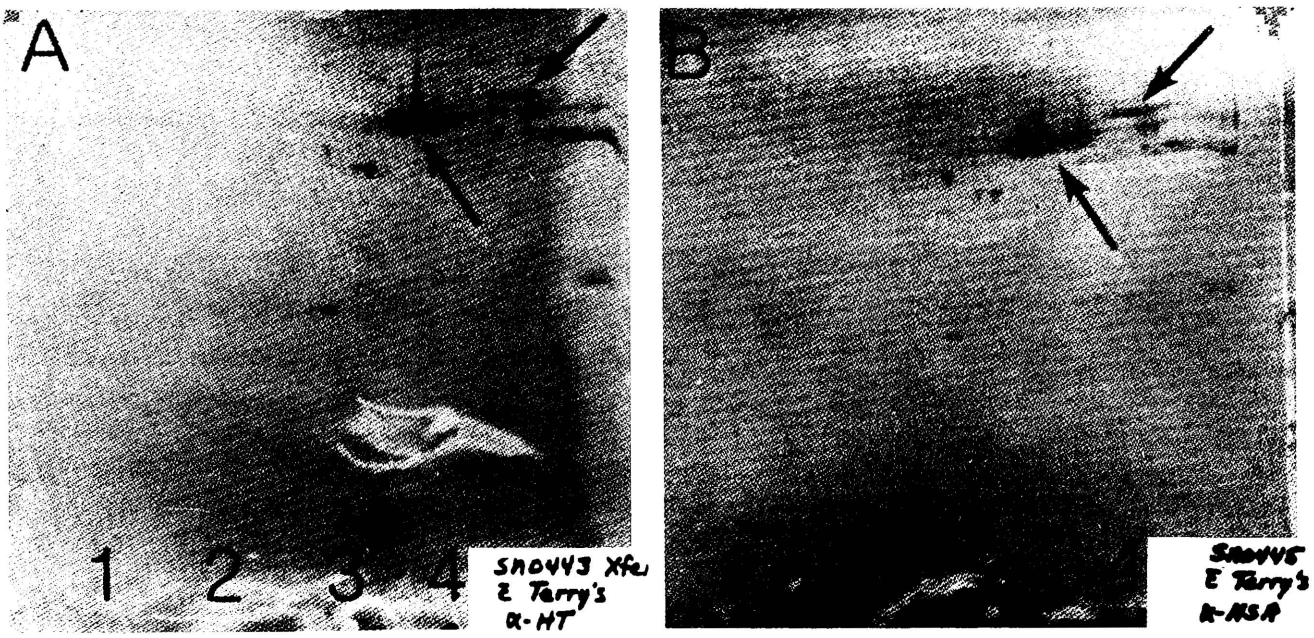

Figure 6. Plasma protein transfers stained with mouse monoclonal antibodies to human transferrin (A) and albumin (B). Neither protein (albumin up arrow, transferrin down arrow) is specifically stained by either antibody as a transferred two-dimensional spot. Both patterns show non-specific background staining as seen on most of the stained plasma protein transfers. Native protein (applied to the nitrocellulose before BSA blocking as small spots) is, however, appropriately stained by the monoclonal antibodies. Four proteins were applied along the bottom edge of the nitrocellulose sheet: (1) albumin, (2) α_1 antitrypsin, (3) haptoglobin, (4) transferrin.

fully capable of providing a "third dimension" analytical parameter in protein mapping studies.

A principal conclusion of the present study is that the vast majority of rabbit anti-human plasma protein antisera tested can recognize their appropriate antigen after two-dimensional electrophoresis in a denaturing system and subsequent transfer to a nitrocellulose substrate. Of the previously identified polypeptides in the system employed, 96 % (24/25) were specifically stained. An additional six proteins were identified for the first time. This result provides a basis for the expectation that a large majority of specific antisera will detect appropriate antigens on a two-dimensional gel transfer. Previous uses of the transfer technique [6, 8], though demonstrating successful antibody staining, have not addressed the question of likelihood of successful detection in the general case. This likelihood now appears to be quite high.

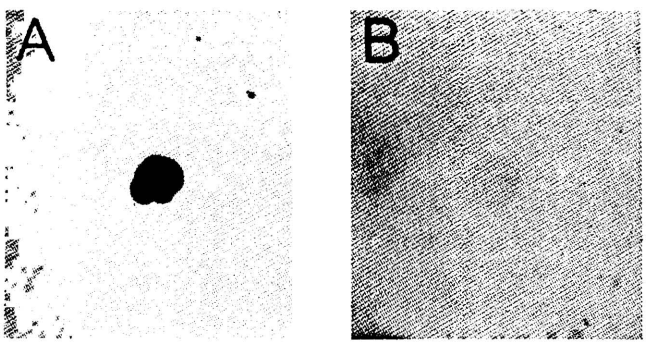

Figure 7. Photographs of small nitrocellulose squares to which had been applied a small circle of human serum albumin (HSA) labeled with [^{14}C] iodoacetamide. The squares were then blocked by incubation in 3 % bovine serum albumin and subsequently treated with rabbit anti-HSA antiserum. At this stage square A was treated with saline (control) while square B was treated with 6 M ammonium thiocyanate. After washing, both squares were treated with anti-rabbit Ig/peroxidase and stained for peroxidase. The circle in B no longer stains, indicating that the original anti-HSA antibody has been eluted by the thiocyanate. However, scintillation counting of the squares revealed 7,326 and 7,250 cpm of [^{14}C] remaining on A and B, respectively, indicating that the originally adsorbed albumin was not removed by thiocyanate.

From the viewpoint of protein folding and specificity, such a result may seem surprising, since it implies that some unique antigenic sites are maintained (or quickly recovered) after prolonged treatment (isoelectric focusing) in 9 M urea, 2 % NP-40 followed by SDS electrophoresis and very strong (virtually irreversible) adsorption to nitrocellulose. In particular the adsorption of the protein to nitrocellulose while still in the form of SDS-protein complexes would seem to allow no opportunity for the reformation of tertiary structural sites. The possibility exists, therefore, that the antibodies staining nitrocellulose-bound spots are directed against specific, short amino acid sequences that do not need to be properly folded in order to be recognized.

Since monoclonal antibodies are highly specific probes for single antigenic sites on antigens and are thus quite useful in the dissection of complex antigenic mixtures [17], it is important to test their ability to bind to antigens after separation in denaturing gels and transfer to nitrocellulose. The three mouse monoclonal antibodies tested were originally selected for strong binding to native antigens adsorbed to polyvinyl chloride microwells. While they bind to their appropriate antigens applied to the nitrocellulose in a native form, they fail to bind specifically to the corresponding transferred two-dimensional spots. This result strongly suggests that the antigenic determinants recognized by these monoclonal antibodies are lost during the denaturing separation (but are not

affected simply by adsorption to nitrocellulose). Further extensive studies will be necessary to determine the proportion of monoclonals directed against such tertiary and secondary structural antigens, but it seems likely to be high. Experiments in this laboratory attempting to use commercial monoclonal antibodies to identify human lymphocyte antigens (β_2-microglobulin, HLA, *etc.*) or to stain several as yet unidentified human urinary proteins on two-dimensional gel transfers have so far been uniformly unsuccessful. Hence the use of monoclonal antibodies to stain spots on two-dimensional transfers may be considered problematical, unless the antibodies are initially selected for the ability to recognize an antigen in a denatured state.

The results with animal antisera confirm a majority of the protein identifications made previously in the human plasma two-dimensional pattern [9], and have extended those results by providing six new identifications. An updated diagram (Figure 5) now shows a total of 37 polypeptides identified. Small amounts of specific antibody to protein spots not yet isolated biochemically may be obtained by ammonium thiocyanate elution of immunoglobulins from spots in a transfer stained with a polyspecific (*i.e.*, anti-human serum) antiserum. Preliminary experiments suggest that such a procedure will be particularly valuable in preparing antibody to a variety of lymphoid cell proteins.

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