4 High-Resolution Two-Dimensional Electrophoretic Mapping of Plasma Proteins

N. Leigh Anderson, Russell P. Tracy, and Norman G. Anderson

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I. Introduction

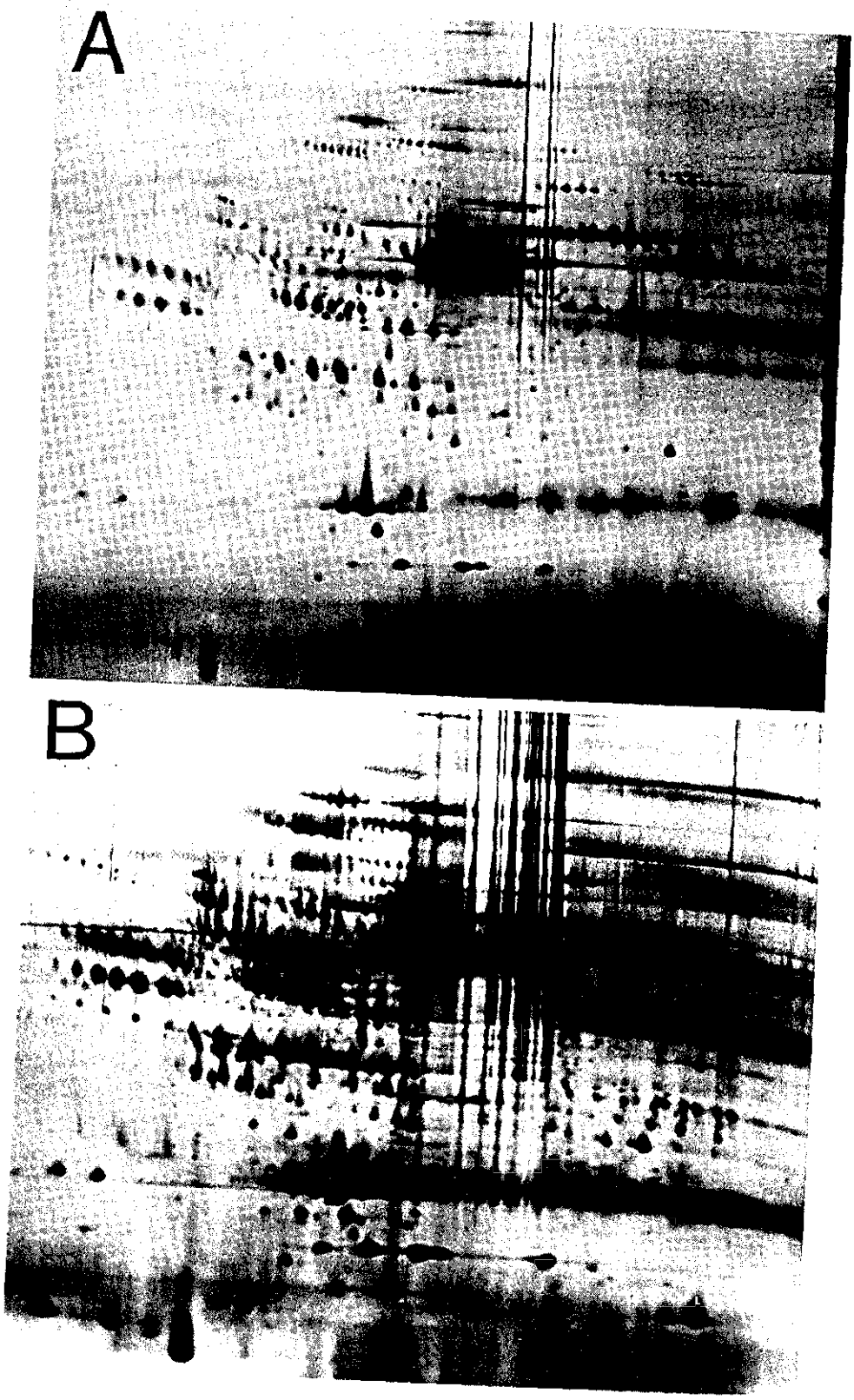
Classical protein fractionation methods have been applied with great success to the isolation and characterization of plasma proteins, with the result, manifested in this and previous editions of volumes in this treatise, that plasma represents the most completely described collection of animal proteins. Nevertheless, there are important limitations to the conventional methods for fractionating such mixtures: first, it is difficult to establish that all proteins present at or above certain abundance limits are indeed being separated and detected, and second, it is difficult to observe and quantitate all of the proteins (including possible novel polypeptides) in a sample simultaneously (i.e., to characterize a sample completely). These difficulties are analogous to the problems encountered earlier in this century in inorganic analysis, where they were largely solved by the introduction of global analytical techniques (e.g., mass spectrometry) capable of resolving and directly detecting all, or a large number, of the possible substances present. The only technique presently available with sufficient resolution to fill a similar role in protein biochemistry is two-dimensional polyacrylamide gel electrophoresis performed under denaturing conditions. In this chapter, we have attempted to bring together the results obtained to date in the application of the technique to plasma proteins, and to provide an updated reference map for use in future investigations.

Two-dimensional polyacrylamide gel electrophoresis, based principally on the method of O'Farrell (1975), has been described elsewhere in considerable detail (N. L. Anderson and Anderson, 1977, 1978; N. G. Anderson and Anderson, 1978). It involves, in the first dimension, a separation by isoelectric focusing in gel rods containing 9 *M* urea and 2% Nonidet P-40 (a nonionic detergent), and in the second (subsequent) dimension, a perpendicular separation in acrylamide gradient slab gels containing sodium dodecyl sulfate (SDS). Proteins are thus separated first by isoelectric point (determined by chemical composition), and second by polypeptide length (roughly equal to subunit molecular mass). These two properties are effectively uncorrelated; therefore, proteins are spread fairly uniformly over the resulting two-dimensional pattern. The use of denaturing conditions in both dimensions is required in order to obtain high resolution: a single charge difference produces a much greater shift in the p*I* of a small protein (or subunit) than a large one (or oligomer), and small differences in polypeptide length are likewise more easily detected by examination of individual polypeptides rather than assembled oligomers. Denaturation also lessens the likelihood of proteolysis during analysis. Although the denaturing conditions used make it generally impossible to assay biological activities after such an analysis, specific antibodies are nevertheless able to recognize appropriate proteins after separation (as described further). The principal purpose of the technique is to provide as complete an inventory as possible of the polypeptides in a mixture and thus to create protein catalogs (N. G. Anderson and Anderson, 1979, 1982).

Other types of two-dimensional electrophoretic techniques have been applied to the plasma proteins, including nondenaturing systems (isoelectric focusing of native proteins followed by pore-gradient electrophoresis, Emes *et al.*, 1975; Manabe *et al.*, 1979, 1981; Felgenhauer and Hagedorn, 1980) and immunoelectrophoresis (which depends on the use of specific antibody for detection, Laurell, 1965). These methods are superior for the investigation of polypeptide associations, but are not as suitable for generating catalogs of protein gene products because of their limited resolution. Therefore, such alternative methods are not included in this chapter; results presented here were generally obtained with the high-resolution two-dimensional (O'Farrell-type) electrophoresis.

II. Two-Dimensional Map of Plasma Proteins

The first high-resolution two-dimensional analyses of plasma proteins were undertaken in 1976, with the aim of demonstrating the usefulness of comprehensive maps of known proteins in the search for genetic variants (N. L. Anderson and Anderson, 1977). At that time, the best protein detection method available for nonradiolabeled proteins was staining with Coomassie Brillant Blue, a procedure capable of revealing a spot containing 50 ng of a transferrin standard (N. L. Anderson, unpublished). Several hundred spots were detected, and these appeared to fall into 50–100 groups. A provisional standard map derived from such a Coomassie Blue-stained pattern served as the basis of plasma protein identification as the number of identified polypeptides increased from about 30 (N. L. Anderson and Anderson, 1977) to 38 (N. L. Anderson *et al.*, 1982). The recently developed silver stains (Switzer *et al.*, 1979; Sammons *et al.*, 1981) allow much more sensitive detection of proteins than does Coomassie Blue, and hence make possible an improved version of the standard map containing approximately twice as many spots (646 in the present map; Figs. 1–10). Such a map is created using a computerized image-analysis system to merge data from a series of gels containing various total loads of plasma and serum proteins from a variety of individuals, so that most prominent polymorphic forms and proteins specific to both plasma and serum are represented. Abundant proteins are observed and located on low-loading gels (0.5 μl of a sample of plasma or serum diluted 1 + 3 with the SDS–Ches mix described in the legend of Fig. 1), and less abundant polypeptides are detected in unobscured regions of high-loading gels (10–20 μl sample; Fig. 1B). These spot patterns are combined in a coordinate system derived from a low-loading gel so that the pH gradient is more nearly linear (less distorted by applied protein). A computer representation of the whole combined pattern is shown in Fig. 2. Isoelectric point and molecular mass standards (N. L. Anderson and Hickman, 1979; Giometti *et al.*, 1980) are used to provide internal markers of these parameters, not subject to errors in systematic measurement (pH measurement) or gel-to-gel comparison. Isoelectric point


A
B

standards used here (shown along the bottom inside edge of each panel) were obtained by sequential carbamylation of rabbit muscle creatine phosphokinase; the zero position indicates the p*I* of the unmodified enzyme, and each sequential leftward (acidic) shift represents the blocking of one additional lysine charge. Molecular mass standardization (Fig. 11) was achieved by using a cubic polynomial fit of known molecular mass versus SDS electrophoretic position for 21 plasma proteins and added rabbit muscle myosin (as a high-molecular-weight marker). The approximate SDS molecular mass scale obtained is plotted along the inside left margin of each panel in kilodaltons. Figures 3–6 are expanded representations of quadrants 1–4 of the whole pattern in Fig. 2, including identifications discussed in Section II,A. Figures 7–10 show the same four panels in which all protein spots are identified by master spot numbers for reference.

A. Identification of Known Proteins

A variety of methods exists for the identification of known proteins in the two-dimensional pattern, and for most of the plasma proteins more than one method has been used (Table I). The most direct approach is the co-electrophoresis of a purified protein with a small amount of serum or plasma to locate the pure protein spots with respect to the most abundant proteins (N. L. Anderson and Anderson, 1977). Immunological methods constitute a second type of direct approach, and are of particular value with regard to plasma proteins because of the availability of a wide variety of antibodies. The use of denaturing conditions throughout the analytical system leads to complete dissociation of antigen–antibody complexes, and has allowed the direct analysis of immunoprecipitates

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Fig. 1. Two-dimensional gel electrophoresis patterns of human plasma. Samples (1.0 μl for panel A; 10 μl for panel B) consisted of 1 part of EDTA plasma (platelets removed) and 3 parts SDS–CHES [2% sodium dodecyl sulfate (SDS), 1% dithiothreitol, 10% glycerol, 50 m*M* cyclohexylaminoethanesulfonic acid (CHES) buffer pH 9.5 in water]. Analysis was performed essentially as described in N. G. Anderson and Anderson (1978) and N. L. Anderson and Anderson (1978) except as noted. First dimension focusing gels were 9.5 inches long and contained a 1:1 mixture of Serva and Pharmacia wide-range ampholytes. Focusing was allowed to proceed for 25,000 V-hr, and then the focusing rods were frozen in SDS equilibration buffer. Second dimension gels were 8 × 10 inch slabs 1.5 mm thick containing an 8–18% linear acrylamide gradient, and were prepared individually by a sequential robot casting 10–30 gels per run. Gels were fixed with sulfosalicylic acid and were silver stained twice by a modification of the method of Guevara *et al.* (1982). Patterns were recorded by contact printing on Kodak XRD direct duplicating film by the method of Harrison (1983). The lower loading (A) shows major spots more clearly (and has an overall geometry less distorted by protein overloading), while many minor spots are only detectable using the higher loading (B). Other loadings ranging from 0.5 to 20.0 μl of both plasma and serum were run under the same conditions. Low-loading (0.5 μl) gels with creatine phosphokinase (CK) charge standards (N. L. Anderson and Hickman, 1979) were used to determine p*I* calibration. Gels are shown with acid to the left and high SDS molecular weight at the top in accordance with standard convention.

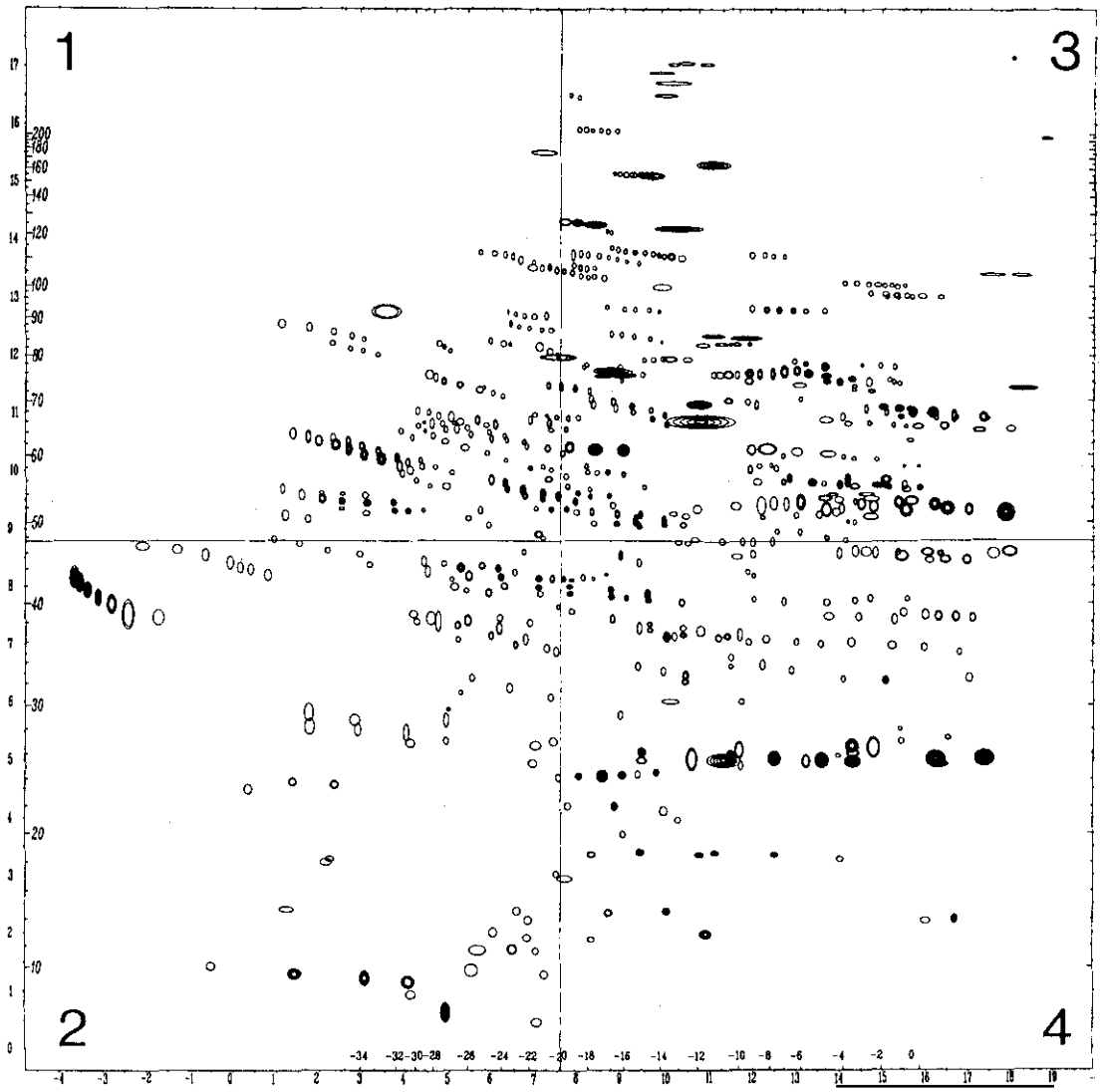

Fig. 2. Computer-generated plot of a composite plasma–serum pattern. The TYCHO system (N. L. Anderson *et al.*, 1981) was used to analyze XRD film copies of gels ranging from 0.5 μl to 10 μl loadings of both serum and plasma. The results were merged in a coordinate system derived from a gel containing 0.5 μl plasma and CK charge standards, then edited. Spots are plotted with x and y widths equal to respective Gaussian half-width spot parameters, and with a number of contours representative of the volume (integrated amount) of the spot. Scales outside the box indicate distances on the base gel in centimeters. Scales inside show position of CK charge standards (in x, the focusing dimension) and approximate SDS molecular mass (along y) as derived from the curve shown in Fig. 11. As in subsequent schematic figures, probable values or identifications are labeled in italic type (as is SDS molecular mass here), and sure values (physically derived) or identifications are labeled in roman type. The four quadrants shown are enlarged in subsequent figures to make labels legible.

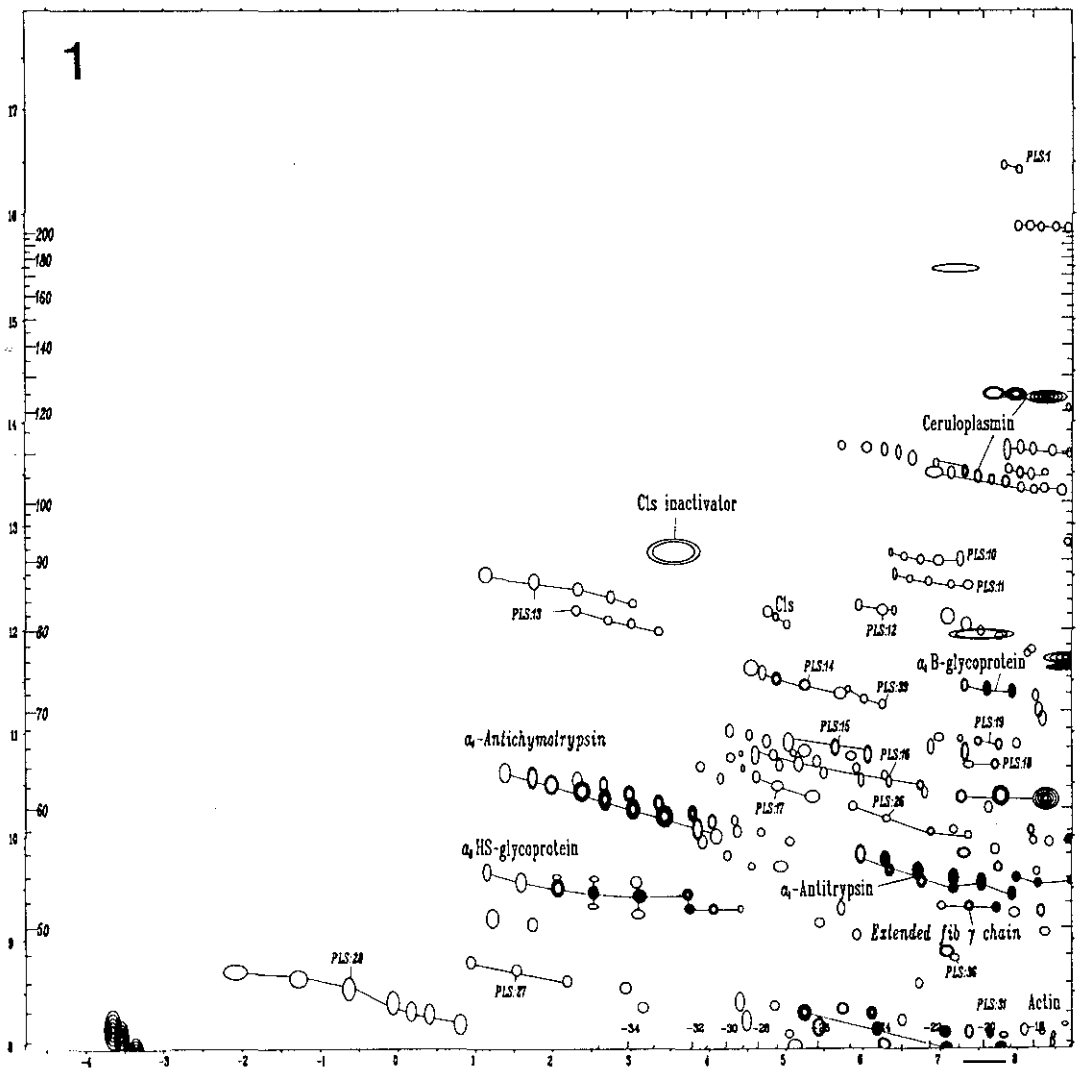

Fig. 3. Identification of proteins in quadrant 1 of the plasma protein map. Axes are labeled as in Fig. 2. Groups of spots are connected on the basis of common identification or probable relationship (based on use of characterization methods described in the text). An as yet unidentified prominent plasma or serum protein is indicated by PLS:*n*.

prepared by mixing human serum with appropriate antisera (N. L. Anderson and Anderson, 1977). A second type of immunological approach makes use of the fact that proteins can be electrophoretically transferred from the two-dimensional gel onto an opposed sheet of nitrocellulose, where they bind noncovalently in a replica of the two-dimensional pattern produced in the gel. These proteins, now exposed on the surface of a nitrocellulose sheet, can react with appropriate antisera, with any bound antibody subsequently revealed by radiochemical or

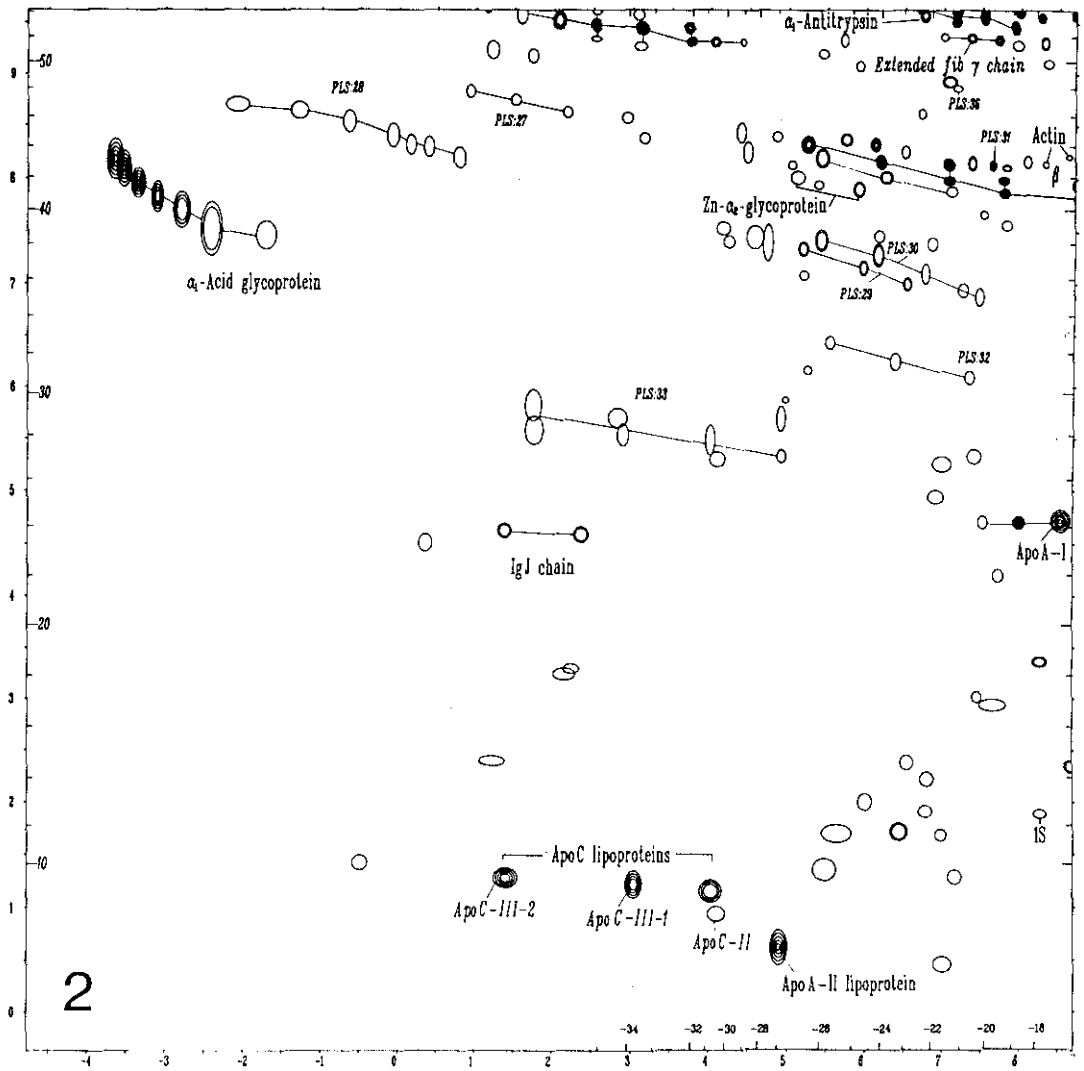

Fig. 4. Identification of proteins in quadrant 2.

enzyme-linked second-antibody methods (Towbin *et al.*, 1979; N. L. Anderson *et al.*, 1982). Studies using a panel of 26 polyclonal rabbit antisera to plasma proteins showed that 95% of the antisera stain the appropriate protein spots on nitrocellulose transfers of plasma proteins despite the fact that all oligomeric structures have been disrupted and that the proteins have been denatured in SDS (N. L. Anderson *et al.*, 1982). Studies using mouse monoclonal antibodies appear much less successful; results indicate that not all (or perhaps even a majority) of the determinants present on the undenatured proteins survive the two-dimensional procedure. The great heterogeneity of the polyclonal response

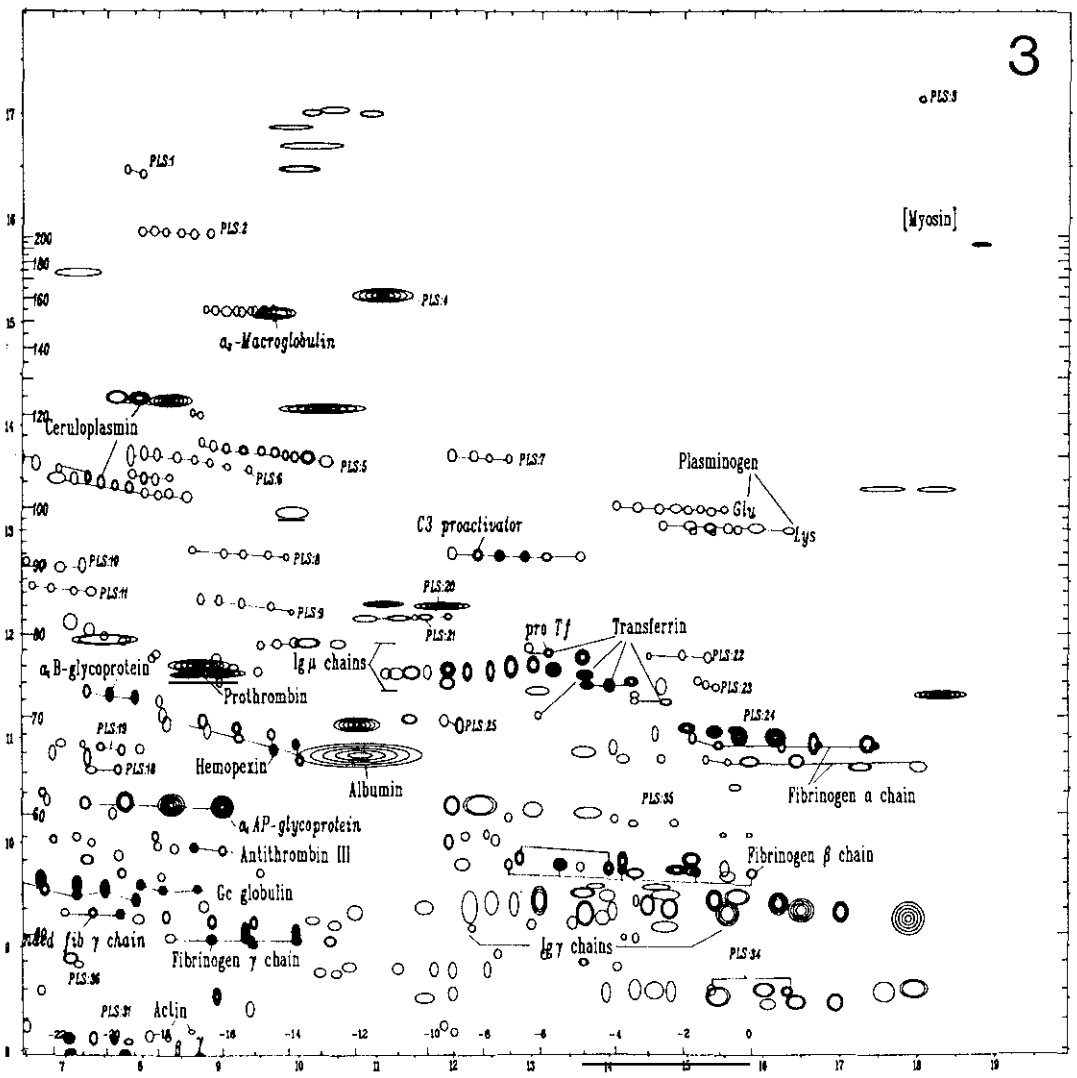

Fig. 5. Identification of proteins in quadrant 3. Myosin (in brackets) is shown for molecular mass standardization; it was not observed in plasma.

leading to generation of classical antisera may therefore be important to the success of identification by the transfer method.

Use of these methods has allowed the identification of 40 gene products commonly observed in two-dimensional analyses of plasma (Table I and Figs. 3–6). Essentially all major plasma constituents and the most abundant leakage products of red cells (Hb) and platelets (actin) have been found. In addition, polypeptide forms of nonstandard chain length (propeptide or cleaved forms) have been found for a variety of proteins, including transferrin, haptoglobin,

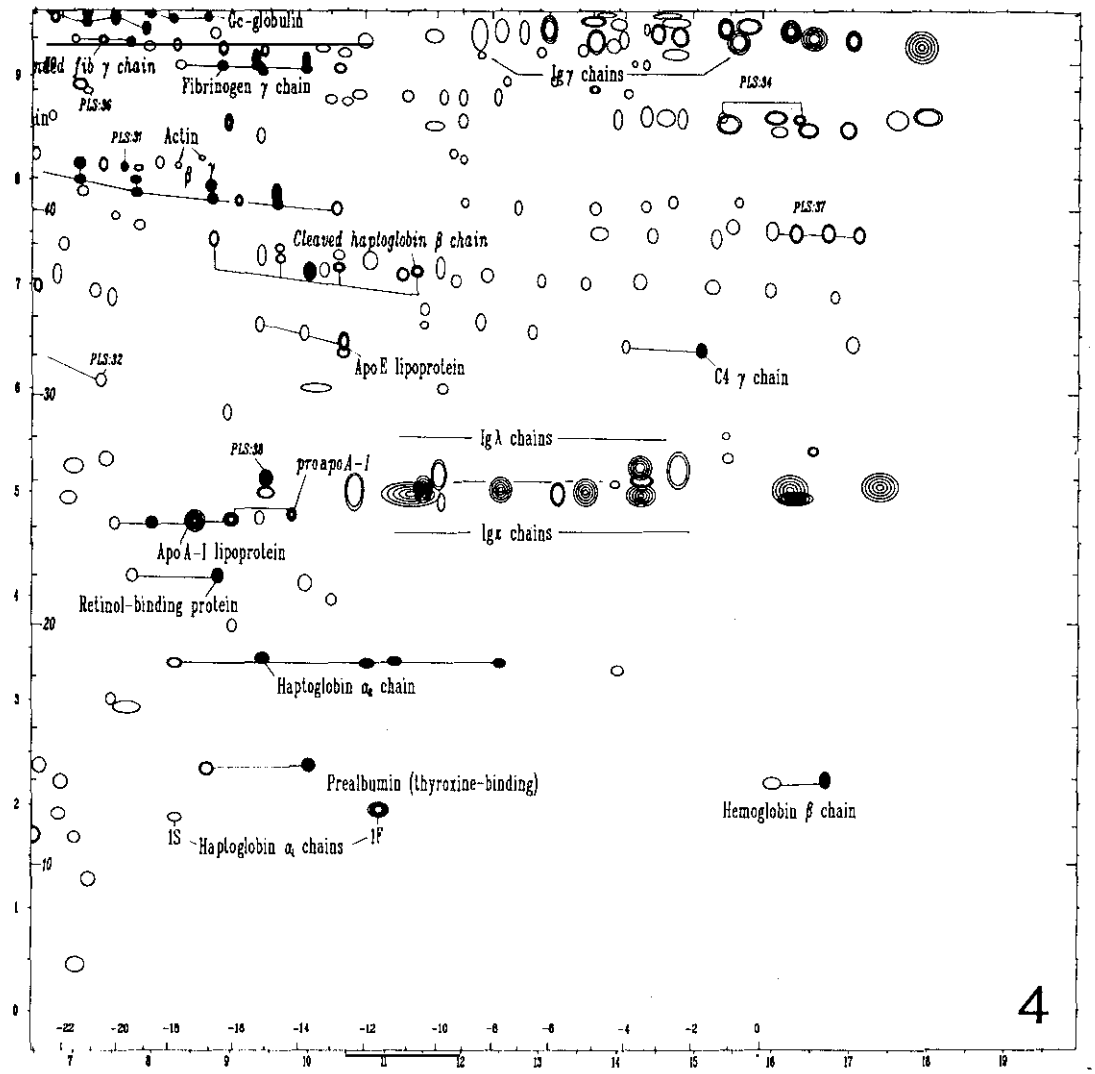

Fig. 6. Identification of proteins in quadrant 4.

ceruloplasmin, α_2HS-glycoprotein, plasminogen, the fibrinogen γ chain, and apoA-I lipoprotein. These proteolytic processing events are described in greater detail in Section III.

A variety of other methods exists for identifying groups of protein spots possessing certain characteristics capable of suggesting or strengthening individual identifications. Among the most direct is the comparison of plasma and serum samples from the same individual (Fig. 12 and Tracy *et al.*, 1982a, Fig. 8). The most striking differences are the disappearance in serum of prothrombin and the fibrinogen α, β, and γ chains, as expected due to the cleavage of the former into active thrombin and the incorporation of the latter into the clot. The

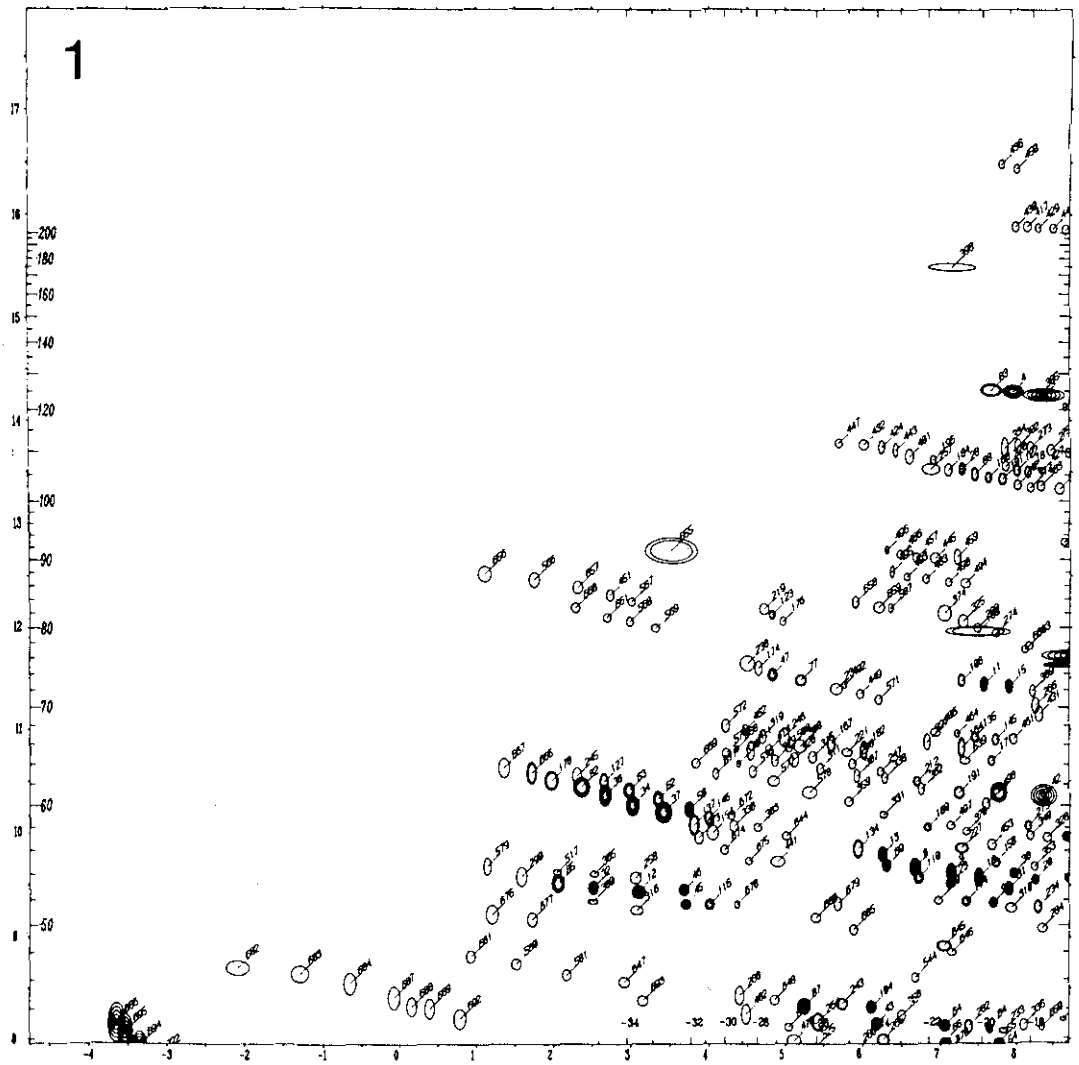

Fig. 7. Identification of spots by master spot numbers (MSN); quadrant 1. A significant number of spots have not been designated by PLS nomenclature, and therefore, are referred to by MSN. These numbers constitute the keys by which data in a computer database may be accessed.

spots identified by Ferguson *et al.* (1981) and Kuyas *et al.* (1982) as an "extended" form of the fibrinogen γ chain also disappear, confirming that these chains are incorporated into the clot with substantial efficiencies (Francis *et al.*, 1980). This extended (γ or γ_B or γ_V) chain has been reported to account for 15–16% of circulating γ chain and to have a C-terminal extension of 2–3 kilodaltons (SDS) (Wolfenstein-Todel and Mossesson, 1980; Francis *et al.*, 1980). Only one major unidentified protein is removed by clotting: PLS:8, a presumed glycoprotein of about 92 kilodaltons (SDS). The studies of Teige *et al.* (1983) suggest that

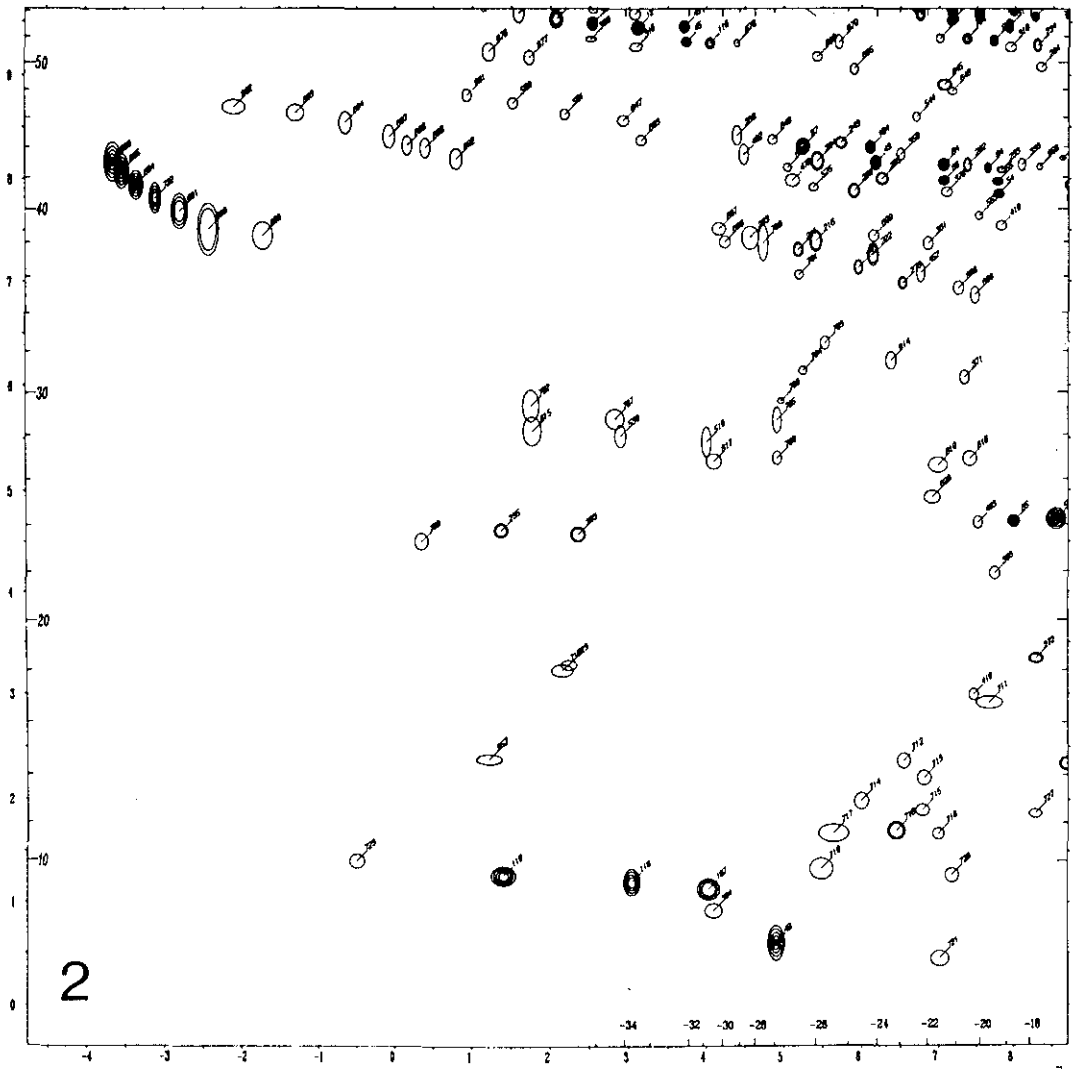

Fig. 8. Master spot numbers; quadrant 2.

PLS:8 may be a γ chain dimer. A number of spots, all as yet unidentified, are generated by clotting and remain in the serum. These include labeled proteins PLS:35 and PLS:36 (which are undetectable in plasma) and spot Nos. 525 and 562 (which can be detected in plasma, but at levels much lower than in serum). A systematic analysis of the solubilizable proteins present in the clot has not yet been carried out; early work showed great heterogeneity, perhaps due to a mixture of proteolysis and transpeptidase products. A further example of the removal of specific spots is the action of added urokinase in removing two trains of spots subsequently identified as the Lys and Glu forms of plasminogen (Fig. 5).

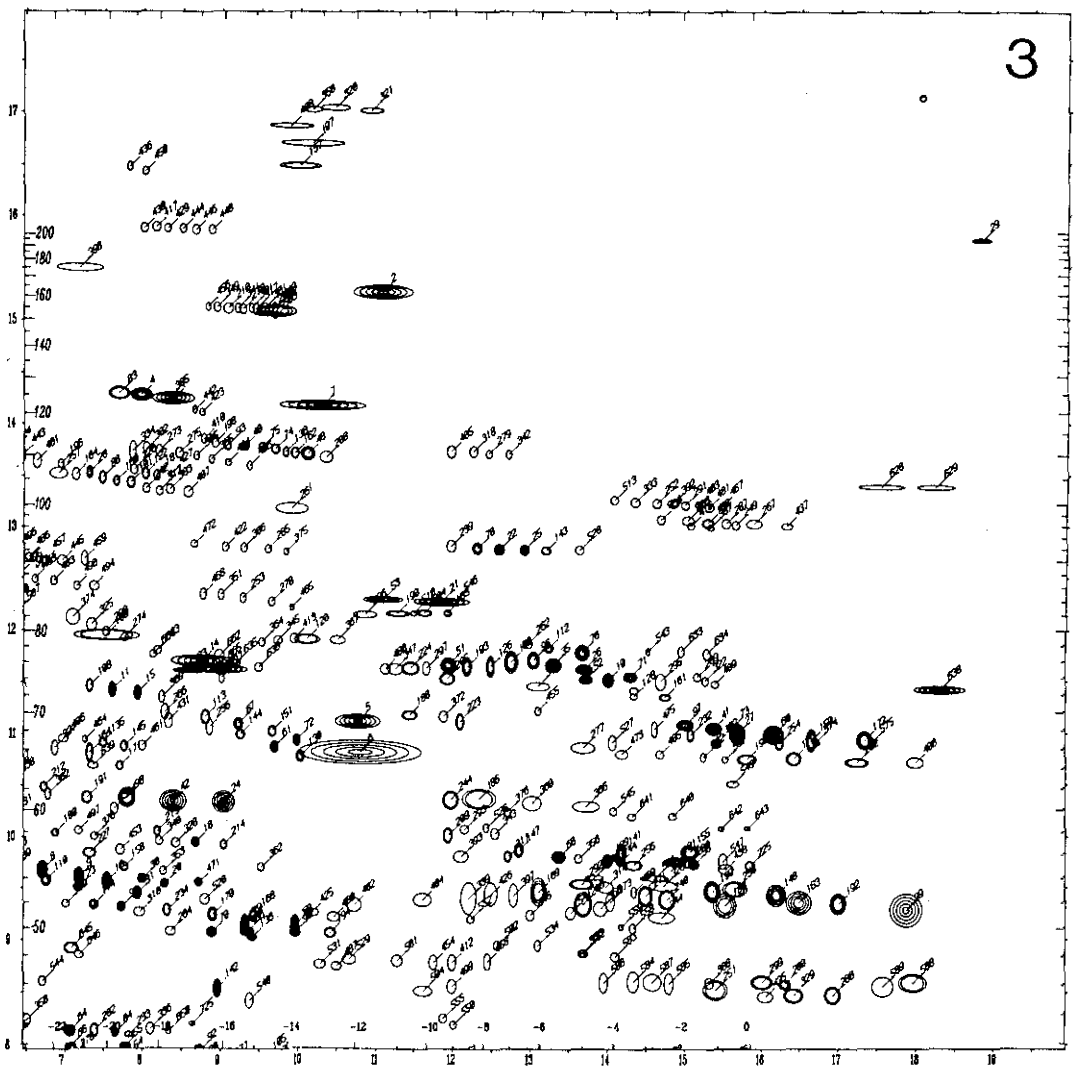

Fig. 9. Master spot numbers; quadrant 3.

Various plasma fractionation methods have also formed the basis of some identifications. High- and low-density lipoprotein samples isolated on the basis of buoyant density have been analyzed and shown to contain primarily apoA-I + apoA-II and apoE + apoC, respectively (N. L. Anderson and Anderson, 1977). The apoB lipoprotein is apparently too large and too hydrophobic to be resolved on two-dimensional gels of this type. The fraction of proteins adhering to Sepharose-bound staphylococcal protein A (Pearson and Anderson, 1980; N. L. Anderson, 1981a) has confirmed the location of various immunoglobulin chains, as described in Section V. Developmental, genetic, and pathologic states provide

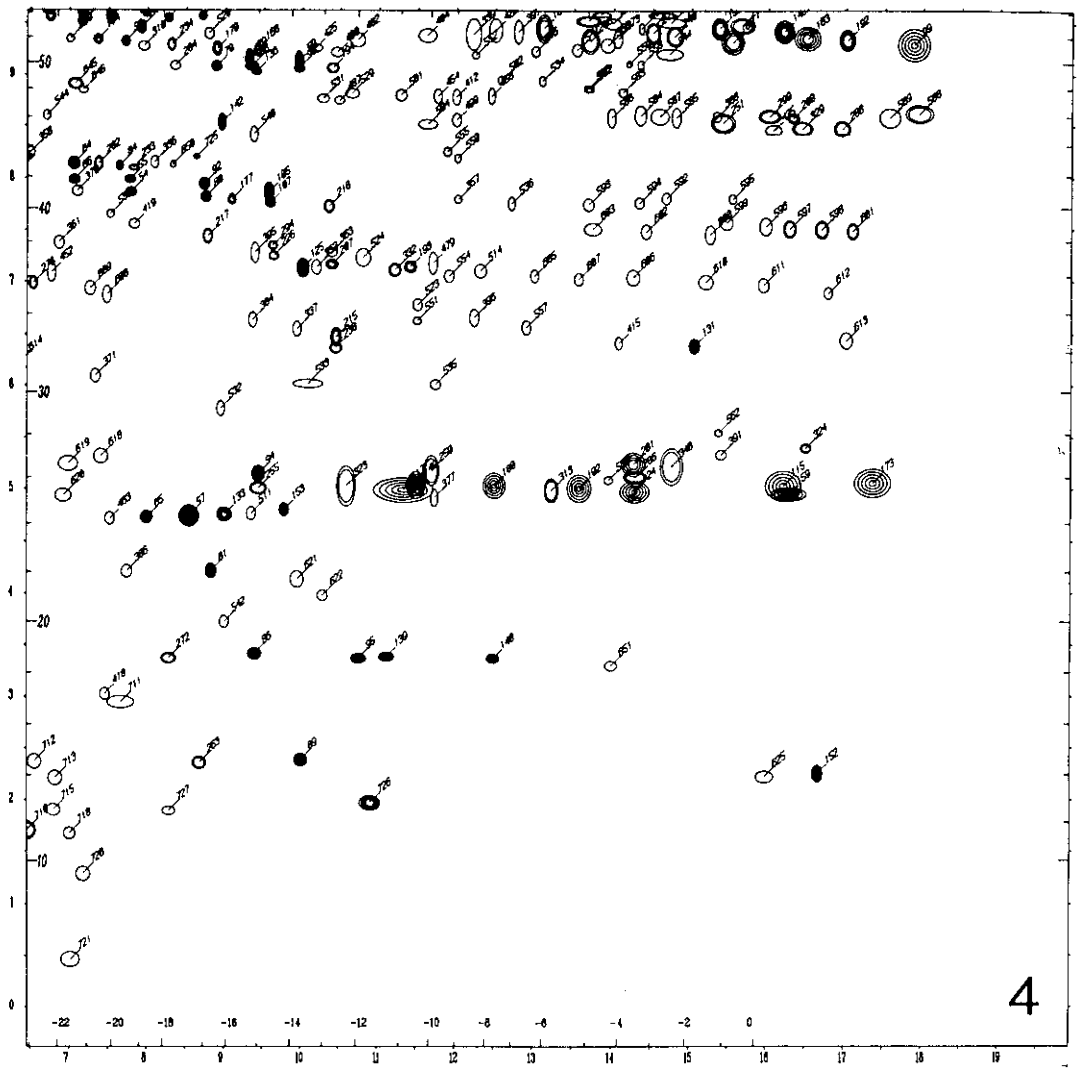

Fig. 10. Master spot numbers; quadrant 4.

important information characteristic of the major protein abnormalities observed. Thus, all haptoglobin chains are missing from cord blood serum and from the serum of ahaptoglobinemic adults. The abundant clonal immunoglobulins produced in human myelomas have allowed identification of the major heavy-chain types, including those not normally visible on plasma or serum patterns (Jellum and Thorsrud, 1982; Tracy *et al.*, 1982b; Section V). Abundant muscle proteins, such as creatine kinase, have been observed in two-dimensional patterns of serum from crush-syndrome patients (Tracy and Young, 1984).

A few proteins show an exaggerated sensitivity to precise conditions of separation in the two-dimensional system, and may thus appear at positions slightly

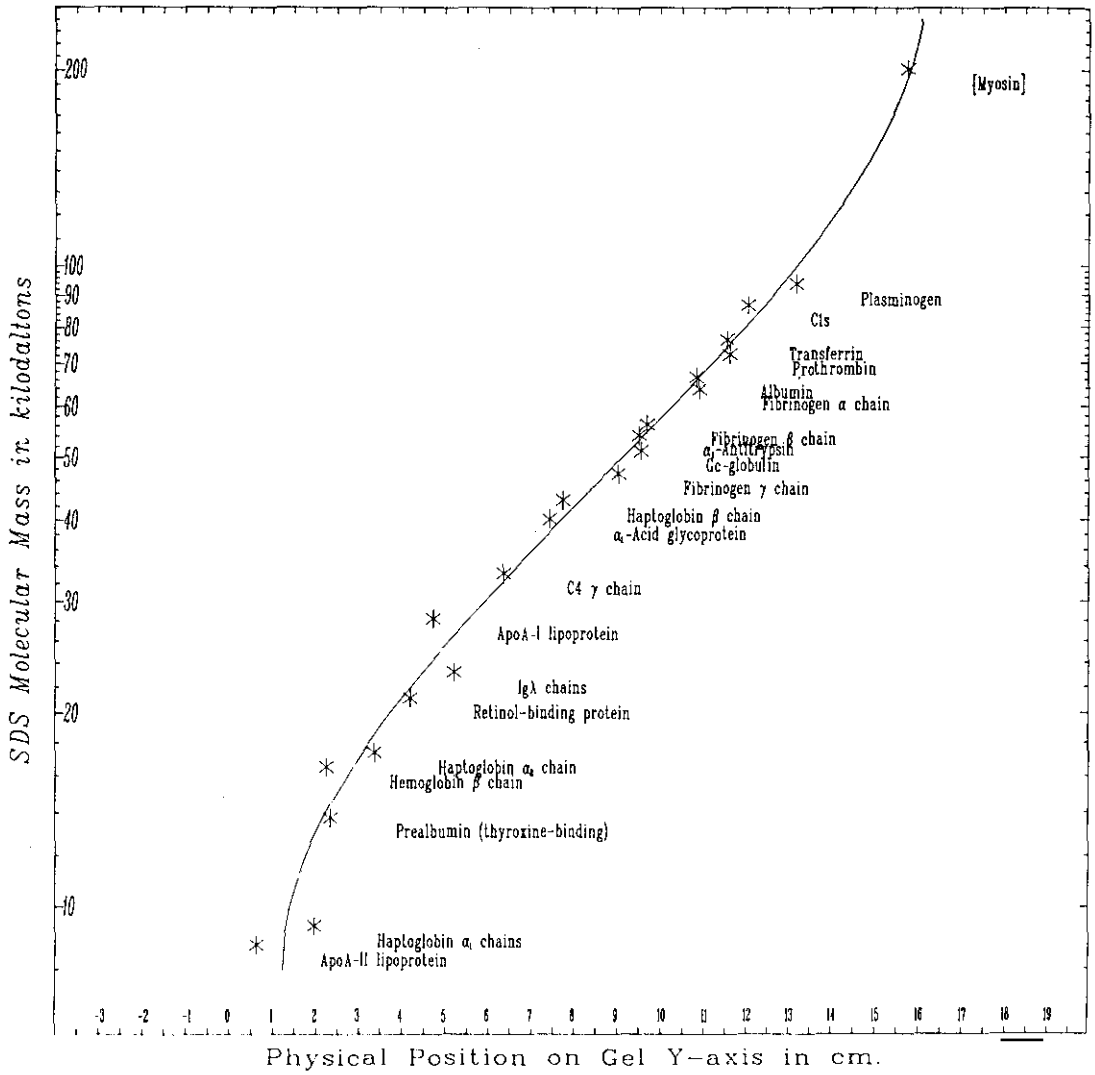

Fig. 11. A plot of *y*-axis (SDS dimension) position versus exact (from sequence) or approximate subunit molecular mass for 21 plasma proteins and rabbit muscle myosin. Spot positions are derived from the standard pattern of Fig. 2. The curve is a third-order polynomial fit to the data; this curve was used to calculate the SDS molecular mass scale of Figs. 2–10. The spread within the data shows that SDS molecular masses are not exact, but appear to be accurate within 5–10% for most plasma proteins. Only values between 10 and 200 kilodaltons (SDS) were considered meaningful.

different from those shown on standard patterns. These include antithrombin III (which sometimes lies over the middle and sometimes nearer the right-hand Gc-globulin spot), α_1-antitrypsin (which can vary in apparent SDS molecular mass, lying above, alongside, or below Gc-globulin), and prothrombin and Cls inhibitor (neither of which focus well, and may therefore appear at variable *pI* values).

TABLE I

Identification of Known Proteins in the Two-Dimensional Pattern[a]

	Protein	Quadrant	x	y	Molecular mass (kilodaltons) (SDS)	Charge standard	Number of spots	Identification references	Pure protein	Ab	Other comments
1.	ApoA II lipoprotein	2	4.9	0.6	<10	−27.0	1	I, V			Presence in high-density lipoprotein (HDL), M_r
2.	ApoC II	2	4.1	0.9	<10	−30.5	1	V			
3.	ApoC III-1,2	2	3.0	1.2	<10	−33.7, <−35.	2	V			C-III 1 and 2 differ by glycosylation
4.	Haptoglobin α_{1F}	4	10.9	1.9	12.8	−11.7	1	I, II	√	xfer, imp	α_{1F} and α_{1S} on basis of sequence
5.	Haptoglobin α_{1S}	4	8.3	1.8	12.4	−17.8	1	I, II	√	xfer, imp	α_{1F} and α_{1S} on basis of sequence
6.	Hemoglobin β chain	4	16.5	2.2	14.0	>0.	2	I			Abundance in red cell lysate, M_r, α chain more basic, presence in complex with haptoglobin
7.	Prealbumin (thyroxine-binding)	4	10.0	2.4	14.6	−13.7	2	I, II, IV	√	imp, xfer	
8.	Haptoglobin α_2	4	10.7	3.3	18.5	−12.0	~5	I, II, IV	√	imp, xfer	Molecular weight of subunit
9.	Retinol-binding protein	4	8.8	4.2	21.9	−16.7	2	Present work	√	xfer	
10.	IgJ chain	2	2.3	4.6	23.7	<−35.	2	II		xfer	Presence in protein A-binding Ig fractions
11.	ApoA I lipoprotein	4	8.5	4.7	24.3	−17.4	4	I, IV, V	√		Presence in HDL
11a.	proapoA I	4	9.8	4.8	24.5	−14.2	1	V		imp	Synthesis by cultured cells
12.	Igλ chains	4	14.3	5.2	26.4	−3.5	Many	I, II		imp, xfer	λ, κ also differentiated by color in silver stain
13.	Igκ chains	4	12.5	5.0	25.6	−7.7	Many	I, II		imp, xfer	Both present in protein A-binding Ig fractions
14.	ApoE lipoprotein	4	10.4	6.3	32.2	−12.6	~4	I	√		Presence in human low-density lipoprotein (LDL)
15.	Haptoglobin β chain	2, 4	9.6	7.7	40.3	−14.7	~11	I, II	√	imp, xfer	Subunit molecular weight
15a.	Cleaved haptoglobin β	4	11.4	7.1	36.4	−10.7	~6	I, II	√	imp, xfer	Reproduces Hpβ microheterogeneity at lower M_r and different pI
16.	Zn-α_2-glycoprotein	2	5.9	7.9	41.1	−24.8	2	Present work	√		High Con A-binding, identified in urine two-dimensional pattern by Edwards *et al.*

19.	Fibrinogen γ chain	3	9.9	9.0	49.4	−13.8	~6	I (mislabeled in ref.), II, IV		imp, xfer	M_r of subunit, removal by clotting
19a.	Extended fibrinogen γ chain	1	7.7	9.3	51.7	−19.6	3	Kuyas *et al.* (1982), IV			
20.	α_2HS-glycoprotein	1	3.7	9.3	51.6	−32.1	~14	I, II, III	√	imp, xfer	
21.	Igγ chains	3	13.1	9.3	51.7	−6.1	Many	I, II		imp, xfer	Presence in protein A-binding fraction, molecular weight
22.	α_1-Antitrypsin	1	7.1	9.5	53.2	−21.3	~9	I, IV	√	imp, xfer	
23.	Gc-globulin	3	8.7	9.5	53.7	−17.1	3	I, II	√	imp, xfer	Observation of the two known genetic forms
24.	Fibrinogen β chain	3	15.1	9.7	54.8	−1.7	~5	I, II		imp	M_r of subunit, removal by clotting
25.	Antithrombin III	3	8.6	10.0	57.3	−17.2	2	I, II, IV	√	imp, xfer	
26.	α_1-Antichymotrypsin	1	3.4	10.2	59.2	−32.7	~15	I		imp	
27.	α_1-AP-glycoprotein	3	9.0	10.3	60.7	−16.0	~3	II		imp	
28.	Albumin	3	10.8	10.8	65.9	−11.9	Several	I, II, IV	√	xfer	Abundance in plasma
29.	Hemopexin	3	10.0	10.8	65.3	−13.7	~8	I, II	√	imp, xfer	
30.	Fibrinogen α chain	3	17.3	10.9	66.6	>0.	~12	I (mislabeled in ref.), II		imp	M_r of subunit, removal by clotting
31.	α_1B-glycoprotein	1	7.9	11.4	72.4	−19.1	3	I	√	imp, xfer	
32.	Transferrin	3	14.0	11.5	74.2	−4.2	~8	I, II, III, IV	√	imp, xfer	
32a.	Protransferrin	3	13.2	11.8	77.5	−5.8	~2	I, III	√	imp, xfer	Reproduces Tf micropattern at higher molecular mass
33.	Prothrombin	3	8.9	11.6	75.1	−16.4	—	II		imp	Removal by clotting
34.	Igμ chains	3	12.2	11.6	75.1	−8.5	Many				Myelomas
35.	C1s	1	5.0	12.0	80.7	−26.7	3	II		xfer	
36.	C1s inactivator	1	3.5	12.7	91.3	−32.5	—	II		xfer	
37.	C3 proactivator	3	12.9	12.7	91.3	−6.6	~6	I, IV	√	imp	
38.	Plasminogen	3	15.4	13.2	99.4	−0.9	~17	I, II		imp, xfer	Removal by urokinase; Glu and Lys forms inferred from known size and p*I* differences
39.	Ceruloplasmin	1	8.4	14.2	124.3	−17.6	~12	I, II, IV	√	imp, xfer	
40.	α_2-Macroglobulin	3	9.5	15.1	153.3	−14.7	~10	I	√	imp	

[a] Positions of the main form of each protein are given in terms of *x* and *y* (in centimeters referred to the master gel coordinate system) and in terms of SDS molecular mass (derived from the curve of Fig. 11) and creatine phosphokinase charge standards (p*I* standards described in Anderson and Hickman, 1979). These values correspond to the scales outside (*x* and *y*) and inside (SDS molecular mass and p*I*) the plots (Figs. 2–10). Publications reporting identification of the proteins and an indication of whether pure proteins or antibody methods (xfer, staining of nitrocellulose transfers; imp, immunoprecipitate) were used are listed. Other bases for identification are listed as comments. Pure proteins used to identify retinol-binding protein and Zn-α_2-glycoprotein were kindly provided by the Behringwerke. Common references are (I) N. L. Anderson and Anderson (1977), (II) N. L. Anderson *et al.* (1982), (III) N. L. Anderson and Anderson (1979), (IV) Goldman *et al.* (1980), (V) Zannis *et al.* (1982a).

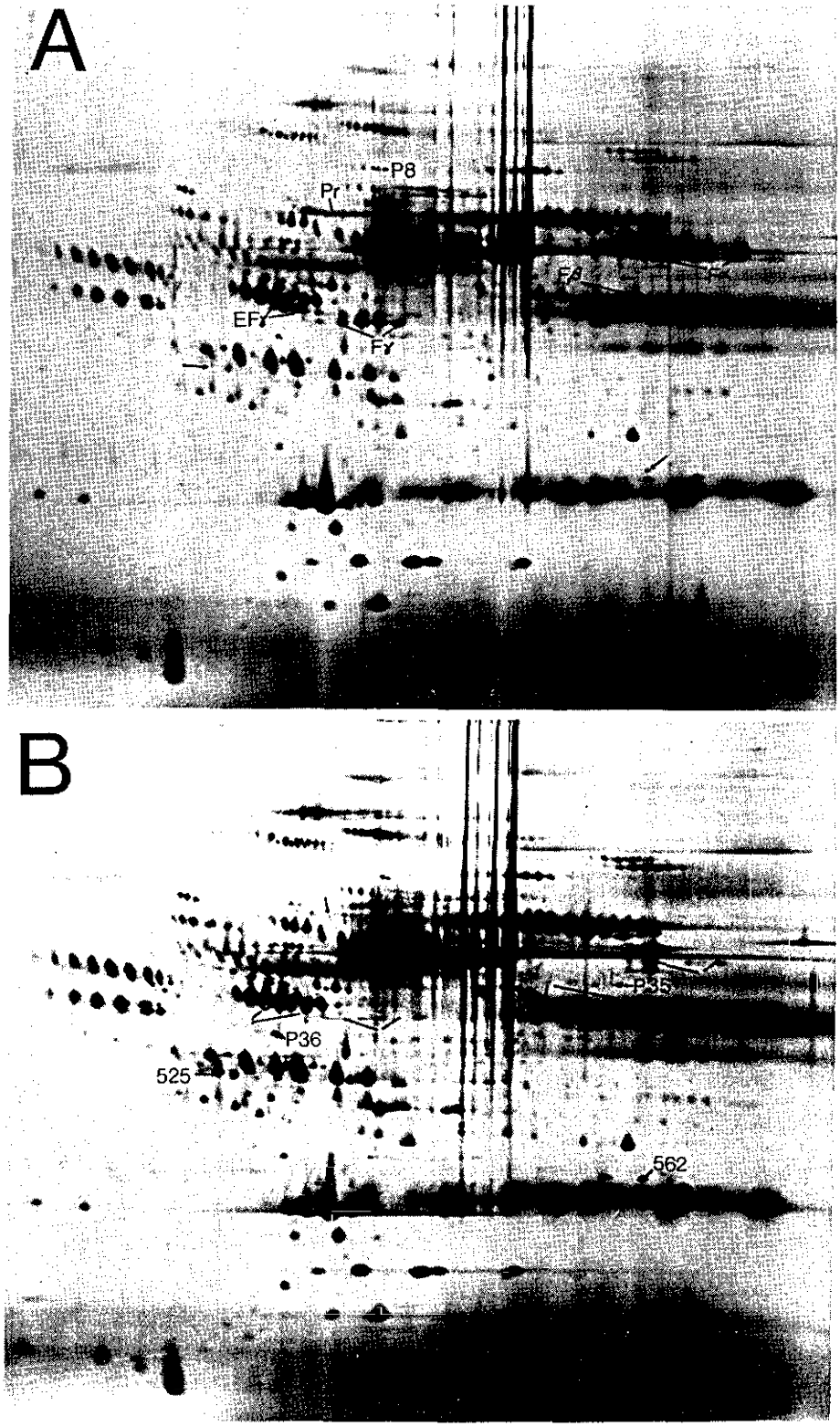

Fig. 12. Comparison of plasma (A) and serum (B) from the same individual. Samples of 3 μl were analyzed as described in the legend to Fig. 1. Six major polypeptides [fibrinogen α (Fα), β (Fβ), γ (Fγ), "extended" γ (EFγ), prothrombin (Pr), and unknown glycoprotein PLS:8 (P8)] disappeared following clotting. Four polypeptides appeared [PLS:35 (P35), PLS:36 (P36), and spot Nos. 525 and 562]. The vast majority of spots was completely unaffected.

B. Characterization of Unidentified Proteins

Although the two-dimensional method was initially conceived as a straightforward analytical approach for quantitating protein abundances, it has more recently been expanded by the introduction of ancillary techniques for differential characterization of protein spots *en masse*. These include determination of partial amino acid compositions, thermostabilities, kinetics of synthesis, modification and catabolism, and regulational control (N. L. Anderson, 1981b). To date, only a limited number of such techniques have been applied to the plasma proteins; however, some tantalizing information has been obtained.

Proteins can be radioactively labeled postsynthetically on cysteine residues by reactions with [^{14}C]iodoacetamide (IAm*, either with or without prior reduction of disulfide bonds, Fig. 13). Sensitivity is roughly comparable to that obtained with Coomassie Blue staining if fluorography is used for detection of incorporated ^{14}C. When plasma proteins are reduced and then alkylated with IAm*, most proteins are strongly labeled (Fig. 13A). Exceptions are apoA-I lipoprotein (which is known to contain no cysteine residues and is completely unlabeled), unidentified protein PLS:31 (which also appears to lack cysteine completely), α_1-antichymotrypsin, and α_1-antitrypsin (which is known to contain "hyper-

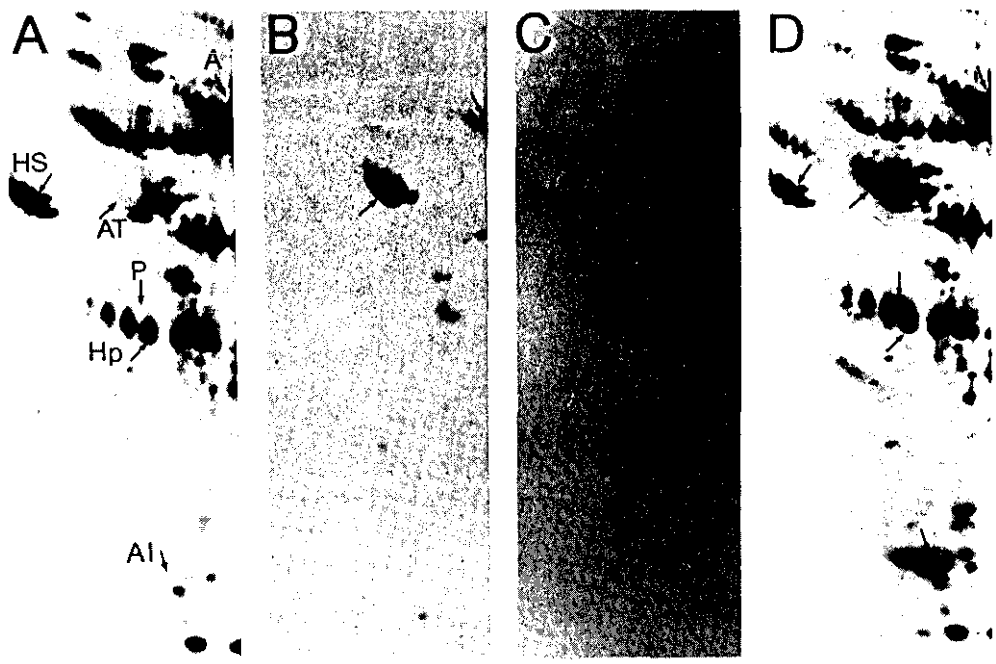

Fig. 13. Sections of two-dimensional plasma protein patterns. (A) Mercaptoethanol-reduced, SDS-denatured plasma after lyophilization and reaction with [^{14}C]iodoacetamide (fluorograph); (B) unreduced, undenatured plasma reacted with [^{14}C]iodoacetamide (fluorograph showing "reactive" sulfhydryls); (C) Schiff stain for carbohydrate (photograph); (D) Coomassie Blue stain for total protein (photograph). α_1-Antitrypsin is indicated by AT, Hp is the haptoglobin β chain, AI is apoA-I lipoprotein, HS is α_2HS-glycoprotein, P is PLS:31, and A is albumin.

reactive'' cysteines, perhaps susceptible to preferential air oxidation during label addition). However, if IAm* is added to plasma proteins without prior disulfide reduction, α_1-antitrypsin is strongly labeled (Fig. 13B), and, with albumin, takes up the bulk of the label. These results confirm that these two proteins bear most of the ''reactive'' sulfhydryl groups of plasma. Several other minor proteins, as yet unidentified in the standard map, also have high relative incorporation in unreduced samples.

Although the determination of glycoprotein carbohydrate content by staining gels with the Schiff reagent has been attempted (Fig. 13C), results are generally poor. Spectacular, but unfortunately irreproducible, negatively stained patterns have been produced using modified Schiff procedures in which proteins were detected with a sensitivity comparable to the silver stain (NLA, unpublished). In general, a far superior technique has been the sequential reaction of plasma protein nitrocellulose transfers with concanavalin A (Con A), antibody to Con A, and finally, a peroxidase-linked second antibody (N. L. Anderson *et al.*, 1982). This procedure can reproducibly detect glycoproteins at levels near or below the sensitivity of Coomassie Blue, and offers the possibility of differentially characterizing glycoprotein carbohydrates by using various lectins. Unidentified proteins found to bind substantial amounts of Con A (and thus containing glucose/mannose carbohydrate) are listed in Table II.

The development of a color version of the silver stain by Sammons *et al.* (1981) has made possible the characterization of proteins by a new, although as yet poorly understood, parameter. In this system, it appears that some overall aspect of protein structure determines silver grain size (and hence color) in a way that cannot be explained simply in terms of carbohydrate or sulfhydryl content or hydrophobicity (D. W. Sammons, personal communication). Nevertheless, individual proteins often display reproducibly distinct colors that distinguish them from neighboring, perhaps interpenetrating, series of spots (Figs. 14 and 15). In the case of known proteins, similar allelic forms (e.g., Gc-globulin types 1 and 2) stain the same color, as do the various carbohydrate-differing variants of major glycoproteins. Proteins that are quite similar, yet differ in a number of amino acids, sometimes yield rather different colors; the κ and λ immunoglobulin light chains, for instance, often can be distinguished on the basis of color. The color silver stain has thus been a major factor in our attempt to deduce the proper relationships between series of unidentified spots.

C. Analysis of Plasma Subfractions and Trace Components

Of course, there are many ways to fractionate plasma for subsequent two-dimensional analysis, including ion-exchange and size-exclusion chromatography, affinity techniques (see Section V,B), ammonium sulfate precipitation, and the classical alcohol precipitation methods. Depending on the method of fractionation, specific and/or trace plasma components may be concentrated and

TABLE II

Major Unidentified Proteins

Protein	x	y	Molecular mass (kilodaltons) (SDS)	pI	Comments
PLS:1	8.0	16.4	>200.	−18.7	
PLS:2	8.8	15.9	>200.	−16.6	
PLS:3	18.0	17.1	>200.	>0.	
PLS:4	11.0	15.2	161.1	−11.4	Strong Con A binding
PLS:5	10.3	13.7	109.9	−12.9	Con A binding
PLS:6	9.3	13.6	107.8	−15.3	Con A binding
PLS:7	12.7	13.7	110.2	−7.1	
PLS:8	9.8	12.8	91.9	−14.1	Removed by clotting, Con A binding, may be dimer of fibrinogen γ-chains
PLS:9	9.9	12.2	83.3	−14.0	Con A binding, possibly C4α
PLS:10	7.2	12.6	90.1	−21.0	
PLS:11	7.3	12.4	86.3	−20.7	Con A binding
PLS:12	6.4	12.2	82.7	−23.4	
PLS:13	3.0	12.3	84.0	−33.8	
PLS:14	5.7	11.4	72.3	−25.4	
PLS:15	5.2	10.8	66.0	−26.5	
PLS:16	6.0	10.7	65.5	−24.3	
PLS:17	5.3	10.4	61.1	−26.1	
PLS:18	7.7	10.7	64.5	−19.6	Con A binding
PLS:19	7.7	10.9	66.6	−19.5	
PLS:20	11.8	12.3	84.0	−9.5	
PLS:21	11.6	12.2	82.5	−10.2	
PLS:22	15.2	11.8	77.2	−1.4	
PLS:23	15.4	11.5	73.6	−1.2	Silver stains red
PLS:24	15.6	11.0	67.9	−0.5	Possibly C3β
PLS:25	12.0	11.1	69.1	−8.9	
PLS:26	6.3	10.2	59.2	−23.7	Possibly thyroxine-binding globulin
PLS:27	2.2	8.6	46.4	<−35.	
PLS:28	−0.7	8.5	45.8	<−35.	
PLS:29	6.5	7.0	35.8	−22.9	
PLS:30	6.1	7.3	37.4	−24.2	
PLS:31	7.6	8.1	42.8	−19.8	Genetic polymorphism; common basic variant at CPK-17. No detectable SH or Con A binding. Likely to be Apo A-IV lipoprotein.
PLS:32	7.3	6.1	30.8	−20.7	Ba^{2+} precipitable
PLS:33	4.1	5.3	26.9	−30.5	
PLS:34	16.3	8.6	46.0	>0.	
PLS:35	14.8	10.2	59.5	−2.3	Appears following clotting
PLS:36	7.1	8.8	48.0	−21.2	Appears following clotting
PLS:37	16.7	7.5	38.8	>0.	
PLS:38	9.4	5.1	26.2	−15.0	
PLS:39	6.2	11.3	70.9	−23.9	

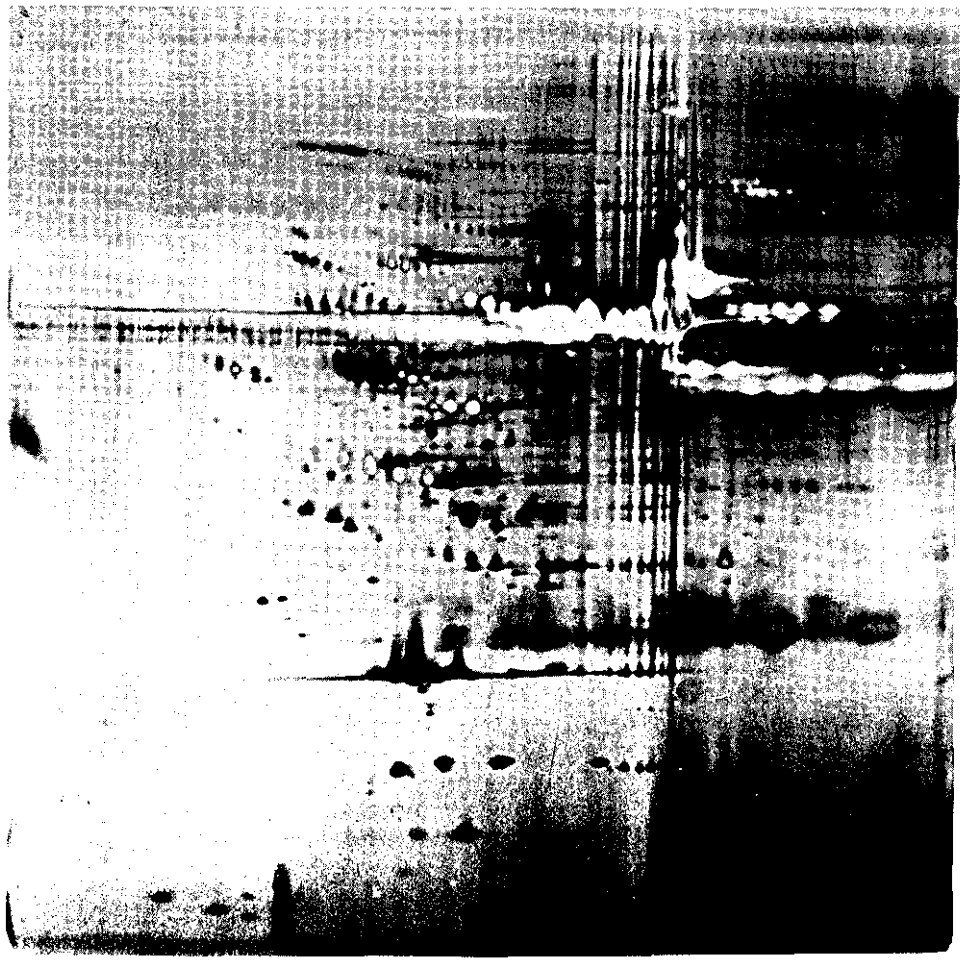

Fig. 14. A two-dimensional separation of platelet-rich pooled human plasma (7 × 7 inch gel system) silver stained using a variation of the Sammons *et al.* (1981) technique. The gel was contact-printed onto Kodak XRD film according to Harrison (1983). Some proteins stain negatively (yellow background), including plasminogen, fibrinogen α, β, and γ, Ig γ and α chains, Gc-globulin, and hemopexin. Others stain very darkly (e.g., α_1-antitrypsin and apoA-I lipoproteins). Such variations are very useful in determining whether relationships exist between spots.

subsequently visualized on two-dimensional gels. In many cases, proteins revealed by these methods are not seen on two-dimensional gels of whole plasma, but must nevertheless ultimately be integrated into the standard plasma map. Interesting relationships to major serum proteins may be revealed by this integration.

An example of plasma subfraction analysis is shown in Figs. 16 and 17. Plasma was fractionated by the standard barium citrate precipitation method (Mann, 1976). The precipitate, containing the various vitamin K-dependent coagulation proteins, is formed by salt complexation with γ-carboxyglutamic acid residues. This precipitate was analyzed by two-dimensional gel electrophoresis

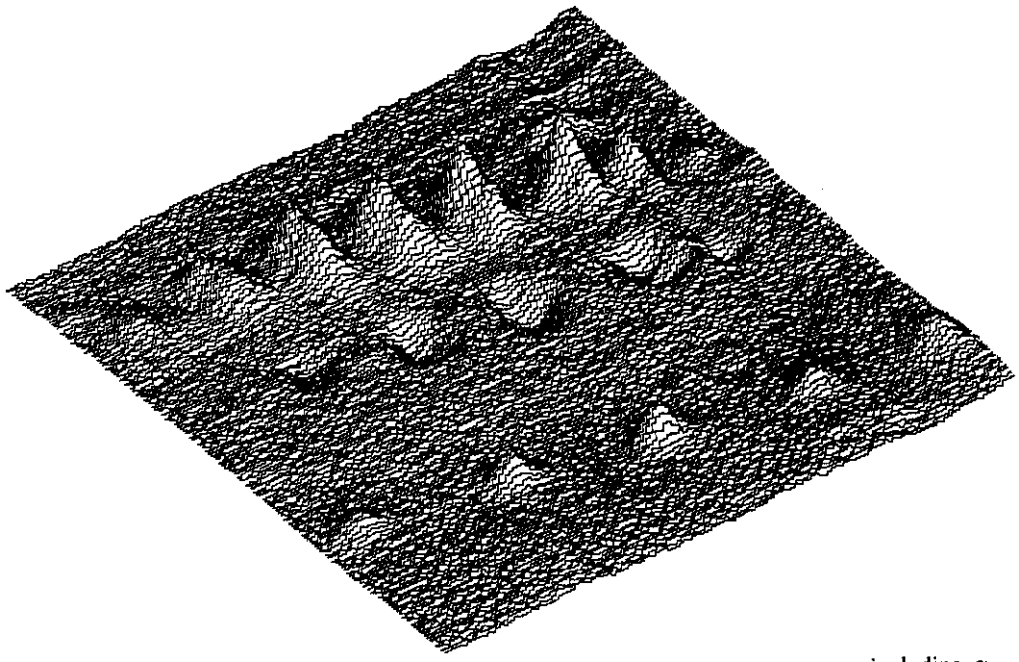

Fig. 15. Density plot of a region from a color silver-stained plasma pattern, including α_1-antichymotrypsin (row at top), α_2HS-glycoprotein (middle row), and PLS:27 (bottom row). All α_2HS-glycoprotein spots stain with negative optical density; all other spots, with positive. α_2HS-Glycoprotein shows negative density in all three visible spectral bands (red, green, and blue).

as described (Tracy *et al.*, 1982a) (Fig. 16). Purified coagulation Factors II, VII, IX, X, protein C, and protein S, along with the barium precipitate, were kindly provided by the laboratory of Dr. Ken Mann of the Mayo Clinic, and were also analyzed electrophoretically. This allowed us to construct the schematic diagram shown in Fig. 17. The schematic drawing also gives the location of several of the major plasma proteins for reference purposes.

This analysis revealed several points of interest. First, as might be expected, prothrombin (Factor II) constitutes the majority of the protein present in the barium precipitate. Also, very few of the major plasma proteins are present, indicating the specificity of the fractionation. The only major plasma proteins present in significant amounts are albumin and apoA-I lipoprotein; lesser amounts of transferrin, IgG, α_1-antitrypsin, Gc-globulin, prealbumin, and several other plasma proteins are present. Second, in a historical context, the difficulty researchers have had in isolating the coagulation factors in pure form is easily understandable from Fig. 16: virtually all the factors on the gel are present in a narrow size and charge range that implies difficulty if standard separation methods are used. Third, although most of the major proteins shown in Fig. 16 may be accounted for, as shown in Fig. 17, several proteins, labeled "a–f" in Fig. 17, are of unknown origin and function. The smaller proteins may represent fragments of the larger factors, or investigation of the larger proteins (e.g., a or b) may reveal hitherto unknown vitamin K–dependent proteins. It is interesting

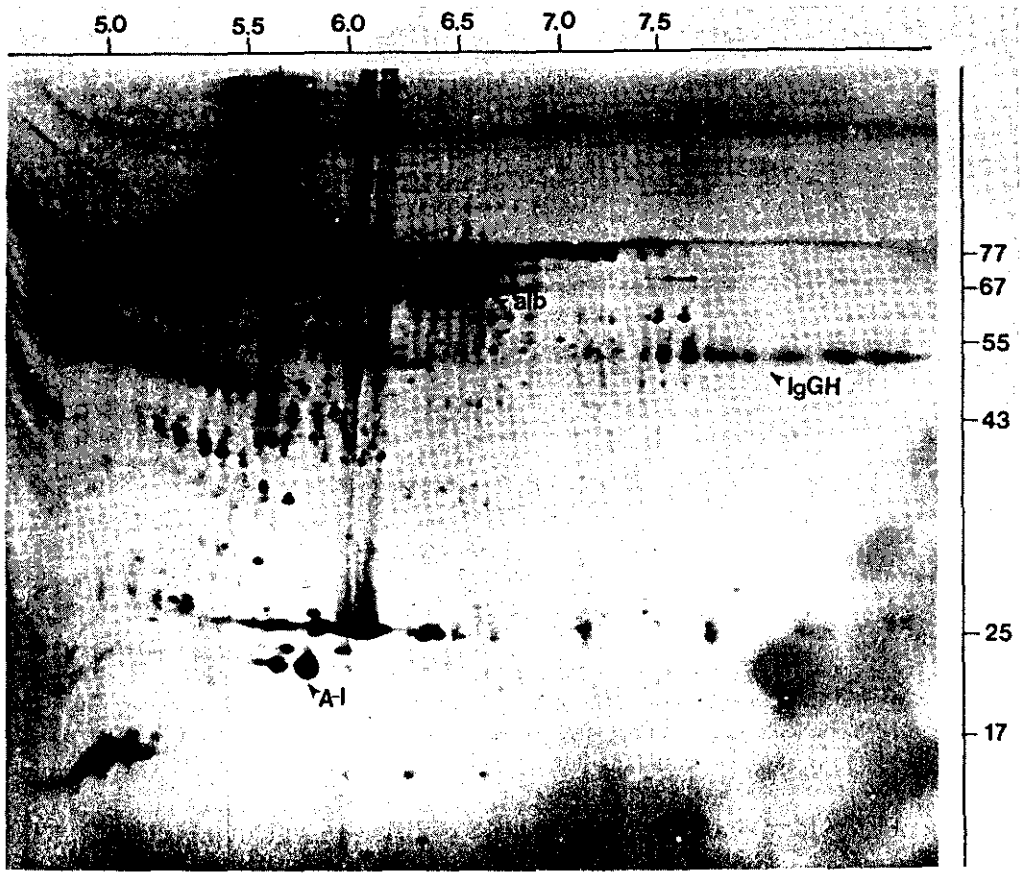

Fig. 16. Two-dimensional pattern of barium-precipitated plasma proteins. Approximately 39 μg of protein was applied to the isoelectric focusing gel. The SDS slab gel was silver stained as described (Tracy *et al.*, 1982a). The horizontal scale is pH and the vertical scale is the apparent molecular mass in kilodaltons. Albumin is indicated by Alb; IgGH indicates IgG heavy chains; A-I is apoA-I lipoprotein.

to note that protein a is almost certainly α_2HS-glycoprotein, a protein known to adsorb tightly to the mineral phase (i.e., the calcium–phosphate matrix) of bone and previously referred to as a "barium" glycoprotein. Many minor proteins present in Fig. 16 have clearly been concentrated from plasma and may also yield interesting results upon further investigation.

D. Plasma Proteins Observed in Other Mixtures

Using two-dimensional electrophoresis in conjunction with any detection procedure that reveals protein generally, it becomes apparent that plasma proteins are major components of most types of sample obtained from higher organisms and of body fluids in particular (reviewed by Dermer *et al.*, 1982, and by Tracy and Young, 1983). The use of fetal bovine and other sera in tissue culture media

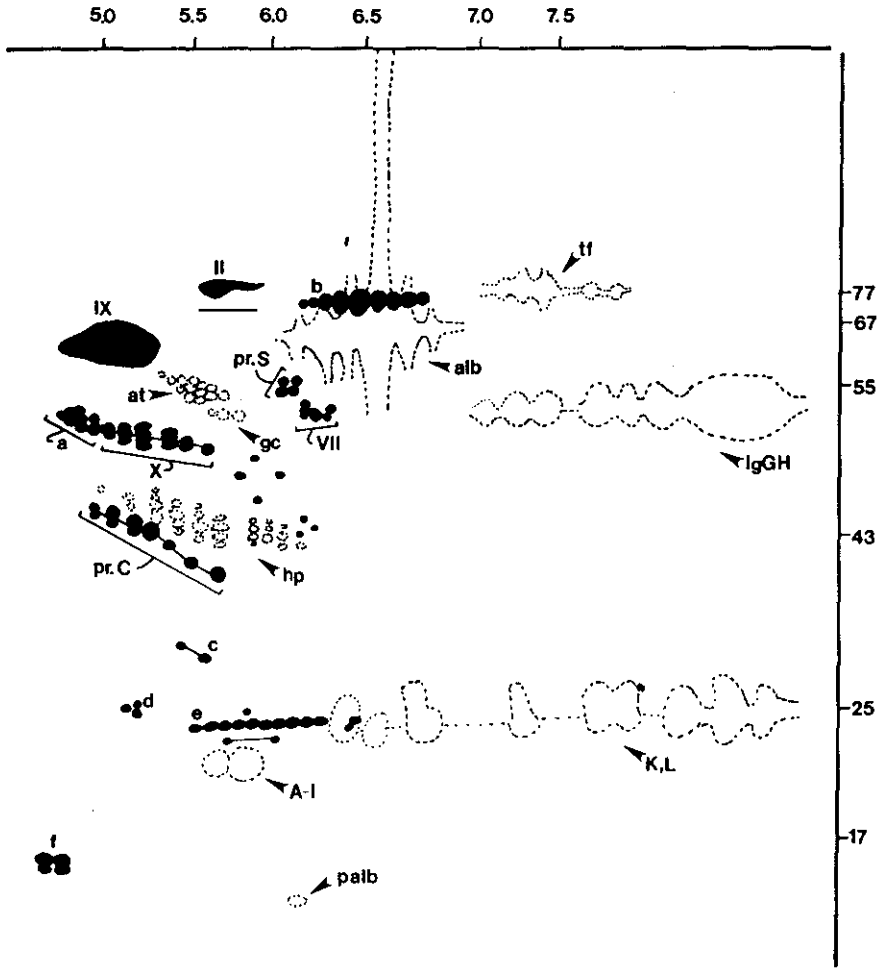

Fig. 17. Schematic drawing of gel analysis of barium-precipitated plasma proteins. Condition and scales are as described in Fig. 16. Additional symbols are: tf, transferrin; K,L, κ, λ light chains; hp, haptoglobin β chains; palb, prealbumin; at, α_1-antitrypsin; gc, Gc-globulin; II, VII, IX, X, respective coagulation Factors; pr.C and pr.S, protein C and protein S; a–f, unidentified barium-precipitated plasma proteins.

results in the presence of serum proteins in most samples of cells grown *in vitro*. It is important to recognize such proteins, and a number of them have been identified in a wide variety of sample types.

Cerebrospinal fluid (CSF) was examined by Goldman *et al.* (1980) using silver stained two-dimensional gels. Cerebrospinal fluid contained at least 26 prominent plasma proteins, and produced a pattern that was generally similar to plasma, with the addition of six groups of CSF-specific spots. Dermer *et al.* (1982) obtained similar results.

Skin blisters, produced by suction or ultra-violet light (UVB) irradiation, yield a fluid that has been examined by Volden *et al.* (1980) and found to be essentially identical to the serum from the same individual, but at approximately one-

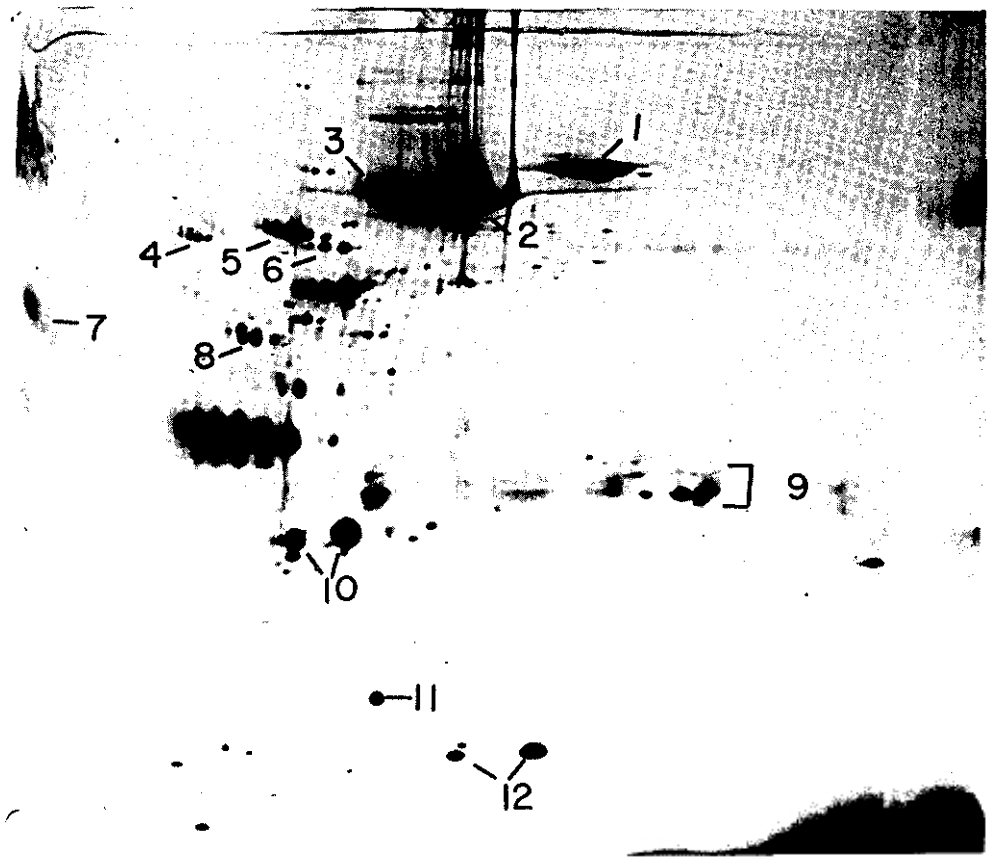

Fig. 18. Two-dimensional pattern of urinary proteins of a normal male (from Edwards *et al.*, 1982b). Identified plasma proteins are transferrin (1), albumin (2), hemopexin (3), α_2HS-glycoprotein (4), α_1-antitrypsin (5), Gc-globulin (6), α_1-acid glycoprotein (7), Zn-α_2-glycoprotein (8), Ig light chains (9), retinol-binding protein (10), prealbumin (11), and β_2-microglobulin (12).

fourth the protein concentration. The prominent cellular proteins, actin and nonmuscle tropomyosin, were observed (although at low levels) in blister fluid, indicating that blister formation was accompanied by protein release from damaged cells.

Amniotic fluid (AF) has been compared with adult and fetal serum by Jones *et al.* (1981) and by Dermer *et al.* (1982). Amniotic fluid contained primarily plasma proteins, appearing to be relatively deficient only in those with assembled molecular masses above about 200 kilodaltons (SDS) (lipoprotein, haptoglobin, α_2-macroglobulin). As expected, haptoglobin was entirely absent from fetal serum. Three groups of nonserum proteins that may be analogous to adult urinary proteins were observed in amniotic fluid.

The human urinary proteins (Fig. 18) have been analyzed in some detail by N. G. Anderson *et al.* (1979a,b) and Edwards *et al.* (1982a,b), and a number of plasma proteins have been identified. The effects of kidney filtration and/or

specific transport mechanisms are evident: high native molecular mass proteins such as fibrinogen, the lipoproteins, haptoglobin, and α_2-macroglobulin are essentially absent, while some smaller proteins such as Zn-α_2-glycoprotein, retinol-binding protein, and β_2-microglobulin are present at much higher levels than in serum. In addition, urine contains a series of nonplasma glycoproteins likely to be of urinary tract origin. One of these, designated the most acid urinary protein (MAUP) (Edwards *et al.*, 1982b) is considerably more acidic than α_1-acid glycoprotein.

Dermer *et al.* (1982) have analyzed pleural effusion fluid (essentially indistinguishable from serum), cervical mucous, and synovial fluid. Both of the latter contain primarily plasma protein, but each appears to have at least one very abundant specific (nonserum) glycoprotein.

Two-dimensional patterns of human seminal plasma (Edwards *et al.*, 1981) contain albumin, transferrin, α_1-antitrypsin, and Gc-globulin together with larger amounts of prostatic acid phosphatase, lactoferrin, and heterogeneous classes of low-molecular-mass and easily proteolyzed basic proteins. Creatine kinase (BB) was also detected at low levels. The presence of intact and apparently unmodified plasma proteins provided evidence that the abundant low-molecular-mass protein spots were not produced by generalized proteolytic activity.

Human saliva has likewise been shown to contain some serum albumin and transferrin, but these are overshadowed by large amounts of salivary amylase (Giometti and Anderson, 1980). High concentrations of secretory IgA make the IgJ chain, secretory component, Igα heavy chains, and κ and λ light chains major proteins of saliva. Major unidentified proteins apparently specific to saliva occur at molecular masses below 20 kilodaltons (SDS) and at about 45 kilodaltons (SDS).

Another high-volume human secretion, milk, has been shown to contain unmodified serum albumin, transferrin, and α_1-antitrypsin, but once again at concentrations much lower than those of the nonserum proteins casein, α-lactalbumin, lactoferrin, and an approximately 85 kilodalton (SDS) glycoprotein tentatively identified as UDPgalactosyltransferase.

Proteins isolated from bovine bone by prolonged dialysis against 0.5 *M* EDTA or by extraction with guanidine-HCl have been divided into about 40 individual groups, 26 of which are present in plasma (Delmas *et al.*, 1983). Major plasma protein constituents of bone include albumin, apoA-I lipoprotein, IgG, IgM, transferrin, α_2HS-glycoprotein, and hemoglobin.

With current detection levels, albumin represents the only plasma protein routinely detected on two-dimensional gels of muscle tissue (Giometti *et al.*, 1979), and distilled-water extracts of human cortical gray matter (Comings *et al.*, 1982) have been shown to contain detectable Igγ heavy and light chains, α_1-antitrypsin, and α_1-acid glycoprotein.

Few plasma proteins have been characterized on two-dimensional gels as

labeled products of the cells that synthesize them. The principal exceptions are the lipoproteins, which have been characterized as products of adult human intestine organ culture (apoA-I; Zannis *et al.*, 1980), of organ cultures of fetal tissue (hepatic and intestinal apoA-I and apoE; Zannis *et al.*, 1982a), of human hepatoma cell lines (apoA-I, apoA-II, apoB, apoC-II, apoC-III, and apoE; Zannis *et al.*, 1981b), and of mouse macrophages (apoE; Basu *et al.*, 1981). The two human hepatoma cell lines investigated by Zannis *et al.* (1981b) were found to secrete isoproteins of apoA-I that are more basic than the major plasma forms and represent proapoA-I. Subsequent work (Zannis *et al.*, 1983) showed that this precursor is longer by six amino acids and more basic by two charges than the mature plasma form, and that the primary translation product preproapoA-I (not detected in plasma) is an additional 18 amino acids longer. The secreted isoprotein forms of a genetically homozygous type apoE were shown to differ by sialic acid only, and are secreted in mature form. ApoC-II, apoC-III-1, and apoC-III-2 (derived from apoC-II-0 by sialic acid addition) are also secreted in mature (plasma) form.

In summary, the ability to observe a large number of plasma proteins by inspection on two-dimensional maps of various body fluids, tissues, and culture supernatants has allowed a rather wide survey of the differential occurrence of these proteins. CSF, AF, blister, pleural effusion, and synovial fluids all contain primarily unselected plasma proteins. The plasma ultrafiltrate excreted as urine contains a population of plasma proteins highly enriched in low-molecular-mass proteins and strongly depleted in those with high "assembled" molecular masses. Fluids produced as external secretions (e.g., seminal plasma, saliva, and milk) show even stronger departures from typical plasma composition, although all contain detectable albumin, transferrin, and other major plasma proteins. Muscle and brain contain plasma proteins, at least partially due to incomplete removal of blood from tissue capillaries, and bone contains a variety of plasma proteins sequestered by methods that are not clear. It seems safe, and perhaps not surprising, to conclude that man is completely permeated by blood plasma and that plasma protein components are thus essentially ubiquitous in clinically obtainable samples.

III. Microheterogeneity

In almost all cases, individual plasma proteins examined by two-dimensional electrophoresis have been found to consist of more than one spot, or molecular form. While the methods for identification described earlier suffice to define the sets of spots comprising each protein, it is desirable to understand the molecular variations that result in the sometimes complex patterns of microheterogeneity that are observed (N. L. Anderson and Anderson, 1977, 1979). Such an under-

standing permits us to organize the unidentified spots into groups likely to represent variations on a few polypeptide themes, and to ask why different proteins exhibit different types of microheterogeneity. From the viewpoint of two-dimensional gel analysis, only protein forms differing in p*I* (by alteration of molecular charge) or SDS molecular mass are likely to be resolved. Thus, in the interpretation of two-dimensional microheterogeneity, we are principally concerned with charge and size changes greater than about 0.1 charge and/or about 0.1–0.5 kilodaltons (SDS).

Although some major cellular proteins may be affected by a rather broad range of postsynthetic chemical modifications, such as N-terminal acetylation (actin), tyrosylation (tubulin), and phosphorylation (vimentin), the principal detectable modifications made to secreted proteins appear to be additions of both charged and uncharged sugars, deamidation, and proteolytic cleavage. A further cause of apparent microheterogeneity in some proteins is genetic polymorphism, a factor that can be analyzed separately through family studies.

These different causes of microheterogeneity are not generally limited in their occurrence to one per protein, and thus one must frequently deal with combinations of different alterations. Fortunately, it appears that a principle of superposition often applies to the interaction of different types of microheterogeneity. If a protein displaying sialic acid microheterogeneity (i.e., whose molecules vary in having n to m sialic acid residues) undergoes partial proteolysis so that a fraction of the molecules have a 10 kilodalton (SDS) peptide of net charge 4− removed from one end, then, in general, we should see the same sialic acid charge train ($m-n+1$ spots long) displayed at two positions, one displaced downward by 10 kilodaltons (SDS) and by four charge shift units to the right (more basic) of the other. An exception to this principle occurs if the removed 10 kilodalton (SDS) peptide contains sites at which some of the sialic acid residues are attached; in that case, the smaller version of the protein cannot repeat the sialic acid charge heterogeneity of the larger form. Nevertheless, such a case can be analyzed in detail, and the contributions of sialic acid addition and peptide removal can be separated if one of the contributing factors is eliminated, as when sialic acid is removed by neuraminidase. Neuraminidase treatment can also be used to clarify the connection between additions of neutral carbohydrate (observed as nonproteolytic alterations in apparent SDS molecular mass) and sialic acid. In this case, there must be an interaction (i.e., lack of superposition) because the sialic acid is usually attached to underlying neutral sugar structures. Hence, the amount of sialic acid and neutral sugar should be correlated.

A second important, though not strictly followed, principle is the equivalence of p*I* shifts (usually termed charge shifts) produced by various p*I*-altering chemical modifications. Addition of sialic acid and most changes in charged amino acids (because of either amino acid exchange by mutation, or deamidation of glutamine to glutamic acid, or intentional carbamylation of lysine) yield an

equivalent alteration in p*I*: the single charge shift. Phosphorylation, acetylation, and a variety of other chemical modifications also produce charge shifts of about the same magnitude, all of them determined by the distance along the protein titration curve that must be traveled (starting at the p*I*) to yield an increase (or decrease) of one charge. Since large proteins (with more amino acids) have more buffering power than small ones, the single charge shift in large molecules is generally smaller. Such a phenomenon is readily apparent in plasma protein patterns (Fig. 1). The exceptions to the charge-shift equivalence rule are relatively few, and involve alterations in some group that is near the middle of its titration curve at the p*I* of the protein. For most proteins, such exceptions are limited to amino acid substitutions involving histidine or to reaction with large substituents such as fluorescein (which shifts hemoglobin chains by about 2.6 charge units). Thus, in general, when two different charge-altering modifications are superimposed, the two collections of spots will be in register in the focusing dimension.

A third principle, implied from the expected statistical nature of glycosylation, is that the abundances of a series of protein forms differing in sugar content will follow some smooth, probably unimodal distribution. Thus, in a train of spots due to simple sialic acid heterogeneity, there should be no "missing" spots in the middle of the distribution. In complex glycosylation micropatterns, the abundances should decrease relatively uniformly in any direction (more or less carbohydrate) away from the statistically most likely form. Departures from such distributions usually signal the presence of other superposed heterogeneities.

A. Examples of Superimposed Heterogeneities

The micropattern of human serum transferrin (Fig. 19) consists of a basic pattern of up to seven spots (as in form II) reproduced at four nearby positions on the gel (I–IV). The variation within form II is likely to be a variation in carbohydrate, because a continuous distribution of material exists, without gaps, centered on the principal spot. The "second row" of spots (upper unfilled spots at 1− and 2− charges) are likely to represent minor forms bearing an additional polysaccharide structure, because they are discontinuously larger than the five other forms and must contain on average more sialic acid (being more acidic). However, the differences among the forms I–IV are likely to be the result of proteolytic cleavage, because a smooth range of intermediate forms is not present and because the molecular mass jumps [3–4 kilodaltons (SDS)] are larger than the approximately 1-kilodalton (SDS) shifts usually associated with neutral sugar structures. The most economical interpretation involves the generation of forms III and IV from form II by removal of a 3-kilodalton (SDS) peptide of charge 2−(II → III), and an overall removal of about 6 kilodaltons (SDS), net charge 3+ resulting in the conversion of II to IV. It is not possible to infer whether these

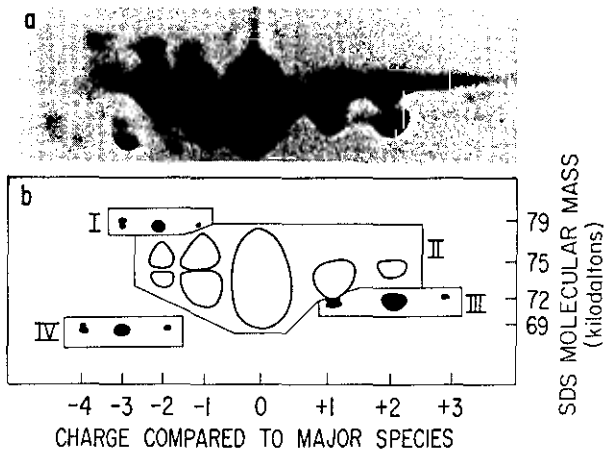

Fig. 19. Microheterogeneity of serum transferrin (from N. L. Anderson and Anderson, 1979). Panel (a) shows a small region of a two-dimensional pattern of immunoprecipitated transferrin. Panel (b) shows a schematic representation of the four major polypeptide forms, each microheterogeneous with respect to carbohydrate.

peptides overlap (and are removed from the same terminus) or are removed from opposite ends. If the latter case is correct, then a simple superposition relationship would require that a form lacking both termini be found located at 1− relative charge and 66 kilodaltons (SDS). However, this form is likely to be too rare to be observed on these gels, being present in a proportion equal to the product of the III:II and IV:II abundance ratios. Form I, which is about 4 kilodaltons (SDS) larger than the main form, cannot be derived from II by cleavage, and it probably represents a precursor of the major form rather than its product. A propeptide of about 25 amino acids with a net charge of 2− would be predicted.

The haptoglobin β chain (Hpβ; Fig. 20a) also shows a series of charge isomers (due to sialic acid) reproduced at a position two charges more basic and about 3 kilodaltons (SDS) smaller than the main form, presumably as the result of removal of a small acidic peptide. In this case, however, a regular, stepwise molecular mass microheterogeneity is apparent within the forms represented by a single sialic acid content and uncleaved polypeptide chain. Thus, a third type of microheterogeneity must be involved, and in this case, it is likely to be a variation in the number of neutral sugar structures attached to the protein. The principal evidence supporting this interpretation is the observation that a desialated (neuraminidase-treated) version of the main form (Fig. 20b) shows four equally spaced bands identical in p*I*, but separated in SDS molecular mass by about 1 kilodalton (SDS). Proteolysis is unlikely to remove three neutral peptides of identical length from one end of the molecule. In addition, it is observed that the higher molecular mass forms are more heavily sialated, indicating that they have more sialic acid attachment sites. Comparison of the desialated forms of the

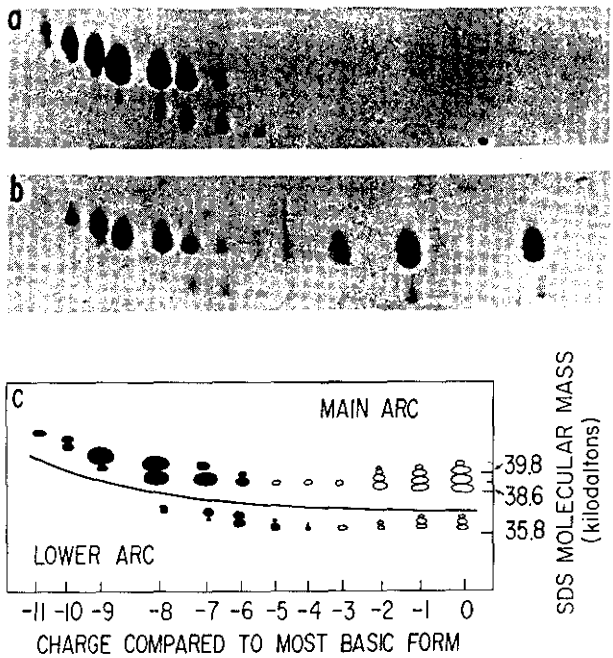

Fig. 20. Microheterogeneity of the haptoglobin β chain (from N. L. Anderson and Anderson, 1979). Panel (a) shows Hpβ chain immunoprecipitated from serum, panel (b) shows neuraminidase-digested (desialated) Hpβ plus undigested Hpβ, and panel (c) shows the results of desialating the main form (main arc) and cleaved form (lower arc). Solid spots are plasma-occurring forms, and open spots are desialation products.

main protein and the proteolyzed product (Fig. 20c) reveals that the p*I*s are almost identical, indicating that the two-charge difference between the original (neuraminidase untreated) large and small forms is not a characteristic of the polypeptide [the removed 3-kilodalton (SDS) peptide must be neutral]. The result of neuraminidase digestion thus suggests that the larger form is two charges more acidic because it carries an average of two more sialic acid residues. Unless polypeptide size influences sialation (which seems unlikely), this result argues that the large form has additional neutral carbohydrate as well, providing more sialic acid attachment sites. A second characteristic of the desialated forms is that, although the larger polypeptide shows three stepwise SDS molecular mass increments [about 1 kilodalton (SDS)], the smaller form shows only two. This difference, interpretable as a deficiency (in the cleaved form) of a neutral sugar structure that normally carries two sialic acid residues, indicates the likelihood of a single carbohydrate attachment site within about 3 kilodaltons (SDS) of one end of the Hpβ chain (in the peptide removed to yield the small form). This is consistent with sequence data showing carbohydrate attachment at Asn-23 [Barnett and Kurosky, quoted by Putnam (1975)]. In addition, the total number of sialic acids in various forms is directly countable, ranging from 6 to 11 for the large (main) form, and from 4 to 9 for the cleaved form.

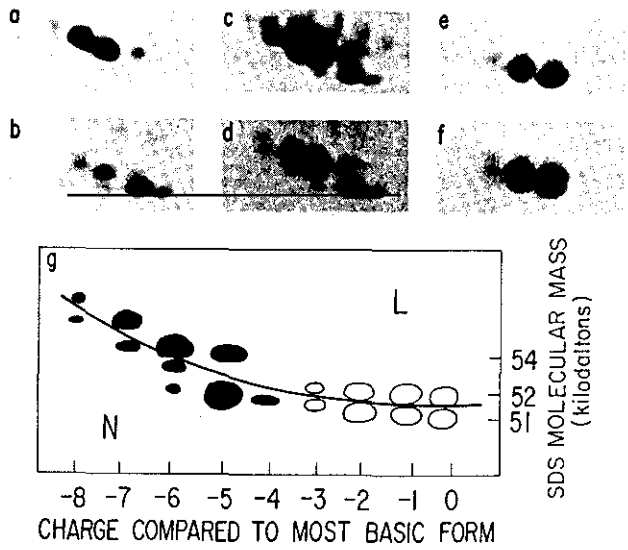

Fig. 21. Microheterogeneity of serum α_2HS-glycoprotein (from N. L. Anderson and Anderson, 1979). (a) Homozygous L form, (b) homozygous N form, (c) mixture of homozygous N and L, (d) heterozygote NL, (e) neuraminidase-treated N form, (f) neuraminidase-treated N and L, (g) schematic showing relationship of N and L (solid spots) and desialated forms (open spots).

B. Examples Including Genetic Heterogeneity

The two prominent genetically determined forms of α_2HS-glycoprotein (N and L) both exhibit micropatterns of four to five spots (Fig. 21). The principal spots of the L form (panel a) lie about 2 kilodaltons (SDS) above and one to two charges more acidic than those of the N form (b). However, the neuraminidase-treated versions of N and L (f) have the same p*I*, and are separated by about 1 kilodalton (SDS) in molecular mass. As in the case of the Hpβ chain, this arrangement suggests that the charge distribution differences between N and L forms are due to differences in sialic acid content, suggesting in turn that an underlying difference in neutral sugar structures is responsible. The observed 1 kilodalton (SDS) molecular mass difference between desialated N and L is consistent with this interpretation. Because the increment in SDS molecular mass expected for a neutral sugar structure is about 1 kilodalton (SDS), any change in polypeptide length must be very small. Therefore, the principal phenotypic difference between N and L appears to be the absence (in N) of one of the two or more neutral carbohydrate attachment sites found in L, a site that usually carries about one to two sialic acid residues. Using antibody-based detection methods, it is possible to detect a further level of heterogeneity in α_2HS (Fig. 22). A replica of the N, L micropattern is observed at low abundance (about 0.1% normal N,L concentration) situated several kilodaltons (SDS) above and three or four charges more basic than the main N,L complex. This material is likely to represent pro α_2HS-glycoprotein.

Fig. 22. Section of a nitrocellulose transfer of plasma proteins stained with antiserum to α_2HS-glycoprotein as described in N. L. Anderson *et al.* (1982). A large arrow indicates the principal L form complex, and a small arrow indicates a similar collection of spots positioned several charges more basic and at higher SDS molecular mass. The latter spots (at about 0.1% the abundance of the main form) are likely to represent a precursor to circulating α_2HS-glycoprotein.

The pattern of α_1-antitrypsin (α_1AT) is more complex (Fig. 23). The M (most common) genetic form consists primarily of an oblique row of three small spots (at the right, basic end) and another oblique row shifted two charges to the left and upward about 1 kilodalton (SDS) consisting of much larger spots. In this case, neuraminidase digestion does not bring the right- and left-hand forms into coincidence, but rather results in appearance of a pair of spots (one charge and a smaller spot two charges to the right; Brown, 1982). Thus, the two-charge difference is likely to be in the polypeptide, and may represent cleavage of a few residues from one terminus to yield the right-hand row. In the case of α_1AT, a considerable number of alleles have been detected. To date, only the positions of M, S, and Z proteins have been compared on two-dimensional gels (Tracy *et al.*, 1982a; Brown, 1982), yielding comparable results. The principal oblique row of spots comprising the S allele is about one charge more basic, and that of Z about two charges more basic than M, in agreement with the substitution of valine for glutamic acid (1+ charge; Yoshida *et al.*, 1977) and lysine for glutamic acid (2+ charge; Yoshida *et al.*, 1976), respectively. The slight apparent molecular mass increments in going from M to S and from S to Z are difficult to explain except as anomalous effects of protein sequence on SDS-binding capacity.

C. Examples of Less Well-Resolved Microheterogeneity

In some heavily glycosylated proteins, molecular mass heterogeneity due to carbohydrate takes a continuum, rather than stepwise, form. Occasionally, very high-resolution gels can resolve stepwise molecular mass increments in the charge isoforms of hemopexin, for instance, but, in general, a column of density at each integrally shifted p*I* is observed. Evidently, the larger the number of carbohydrate attachment sites, and the more varied the types of sugar structures

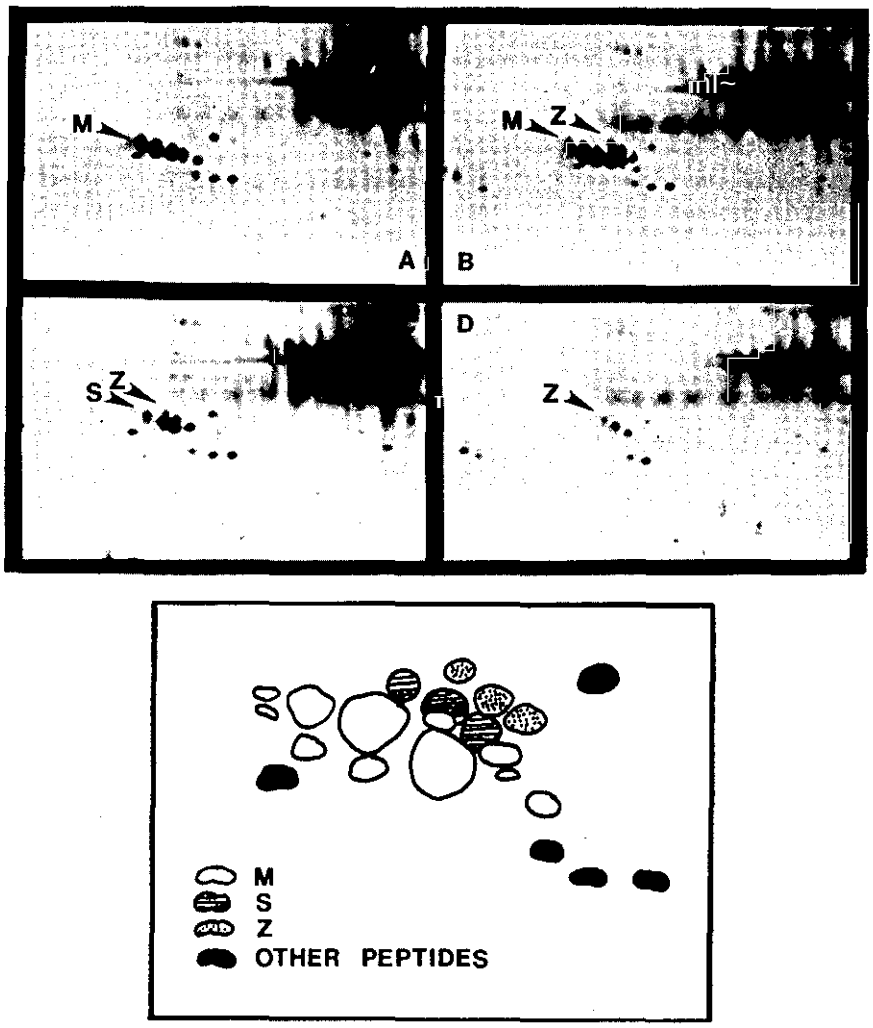

Fig. 23. Regions of two-dimensional patterns of sera showing α_1-antitrypsin types: (A) MM, (B) MZ, (C) SZ, (D) ZZ. Schematic shows the relationship between M, S, and Z polypeptides (from Tracy *et al.*, 1982a).

that can be attached, the greater the number of possible microvariants, and hence the poorer the chances of resolving such heterogeneity. Two extreme cases are shown in Fig. 24, α_1-acid glycoprotein and MAUP. Each spot in the α_1-acid glycoprotein chain (separated by single charge shifts due to sialic acid addition) is much larger than an equivalent nonglycoprotein spot. Each contains substantial unresolved microheterogeneity. The MAUP is a protein so acidic that sulfation could be considered as a source of heterogeneity. Up to ten oblique rows of 5–15 spots fuse to yield a pattern of about 12 almost horizontal bars. The interpretation of such a level of heterogeneity is not possible without detailed knowledge of the modifications involved.

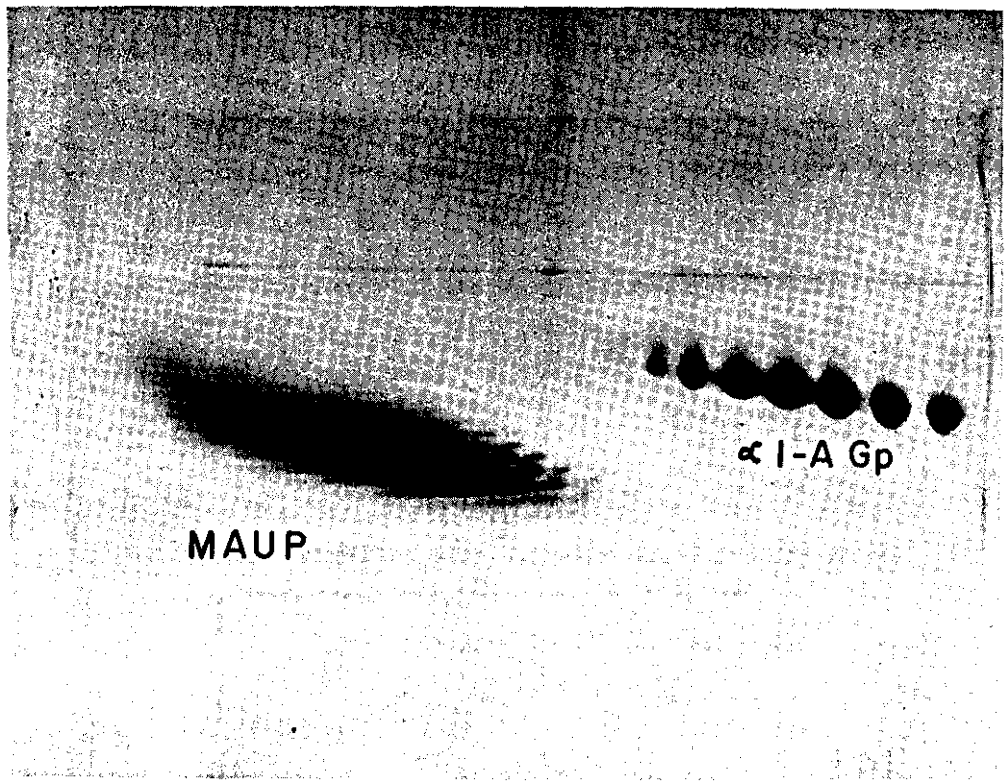

Fig. 24. Two-dimensional pattern of acidic proteins from human urine (from Edwards *et al.*, 1982b). α_1-Acid glycoprotein is α_1A-Gp and the most acid urinary protein is MAUP.

In summary, the microheterogeneity of individual glycoproteins on two-dimensional gels has proven, in a number of cases, to be interpretable. The interpretations can provide testable hypotheses regarding proteolytic cleavage events, and regarding levels and types of glycosylation. Although experience to date with classic endoglycosidases has not been very encouraging, more effective sugar-removing enzymes may substantially improve the interpretability of even complex heterogeneities.

D. Other Examples of Proteolytic Processing

Several proteolytic cleavage events in addition to those already discussed are evident in the plasma two-dimensional pattern. Two forms of plasminogen, the larger and more acidic having an N-terminal glutamic acid and the other an N-terminal lysine (Robbins *et al.*, 1975), are routinely observed at SDS molecular masses of about 99.4 and about 95.8 kilodaltons, respectively (N. L. Anderson and Anderson, 1977). Ceruloplasmin appears as a poorly focused mass at about 124 kilodaltons (SDS) with a smaller, apparently more acidic form that is well

focused (a series of resolved small spots indicative of sialic acid related charge heterogeneity) at about 103 kilodaltons (SDS). These forms are likely to correspond to the 130- and 116-kilodalton (SDS) forms observed by Kingston *et al.* (1977), who used a different system of molecular mass standardization. ApoA-I lipoprotein provides an example (mentioned previously) in which the identity of a circulating propeptide has been rigorously established (Zannis *et al.*, 1980). ProapoA-I is two charges more basic than the predominant form and six amino acids longer. ApoA-I is apparently unusual in that the pro form is the major secreted product.

IV. Genetic Polymorphism

A. Detection of Variants and Average Heterozygosities

Because of its ability to resolve large numbers of proteins, two-dimensional electrophoresis can be used to search for genetic variants at a large number of loci simultaneously. Expectations of such a use prompted the initial two-dimensional analysis of human plasma (N. L. Anderson and Anderson, 1977), and motivated several groups to compare the two-dimensional patterns of small numbers of human cell lines in an attempt to estimate the average heterozygosity occurring over a larger sample of human loci than could before have been examined (McConkey *et al.*, 1979; Walton *et al.*, 1979; Smith *et al.*, 1980). The latter studies yielded average heterozygosities of about 1% or less, results that are clearly at variance with a value of 6.3% obtained in the classic studies of Harris (1980) on plasma proteins and red cell lysate enzymes.

Geneticists have not been quick to accept the results of the two-dimensional studies of cellular proteins over the patiently acquired blood protein data (obtained mainly by starch electrophoresis), and in part, this caution could be likened to wisdom. Although the results raised questions about the reliability of two-dimensional gels and their ability to detect variants, they also prompted some investigators to question the genetic equivalence of plasma and typical cellular proteins. It seems fair to suggest that the plasma proteins, and to some extent the soluble red cell proteins, are designed to fill simple functions requiring rather few intermolecular interactions. An overriding necessity is for the proteins to remain soluble, even when denatured. Attached carbohydrate clearly helps this cause, making plasma proteins far more difficult to precipitate by heat denaturation than average cellular polypeptides. Therefore, it could be supposed that a large proportion of amino acid alterations taking place in the surface residues of plasma proteins may have little or no effect. The population of variants of hemoglobin (another protein engineered for solubility) examined from a structural viewpoint by Perutz and Lehmann (1968) tends to support this

view; changes in surface residues frequently had no serious functional effect, the sickle-cell mutation constituting one obvious exception. However, the set of proteins found in nucleated cells contains at least some proteins whose entire sequences are very highly conserved (such as the histones) or whose precise isoelectric points have been conserved throughout the mammals (such as the β and γ actins; NLA, unpublished observations). McConkey *et al.* (1979) suggested, on the basis of the very low estimated heterozygosity found in human cell lines, that mutations of abundant cellular proteins may often be dominant lethals, and that mutations in plasma proteins may produce less effect. A controversy thus erupted between those who chose to fault the two-dimensional method for observing too few genetic variants, and those who felt that variations in heterozygosity between different classes of proteins were to be expected.

Recently, the question of the ability of the two-dimensional gels to resolve variants detected initially by starch electrophoresis has been addressed directly. Wanner *et al.* (1982) used two-dimensional electrophoresis to test 17 allele products at five loci (carbonate dehydratase I and II, β-globin, albumin, and mouse pancreatic amylase), and were able to separate all but one of the proteins. Therefore, they concluded that "2-D gel electrophoresis can resolve more than 90% of the variants originally detected by one-dimensional (1-D) electrophoresis." Rosenblum *et al.* (1983) then investigated plasma protein heterozygosity using two-dimensional electrophoresis, obtaining a value of 6.2% ± 0.7%, in striking agreement with Harris' (1980) results using one-dimensional electrophoretic screening. Although studies of both plasma and nucleated cellular proteins have not yet been completed in one laboratory, thereby leaving open the question of interlaboratory differences in variant detection efficiency with two-dimensional gels, the results available to date support a significantly (perhaps 10-fold) higher average heterozygosity in plasma proteins as compared to abundant proteins of nucleated cells. Plasma proteins may thus represent an atypical (and perhaps unique) subset of human proteins from genetic and evolutionary viewpoints.

B. Plasma Protein Polymorphisms Observed on Two-Dimensional Gels

The most common polymorphisms evident in two-dimensional patterns of plasma proteins are those involving the haptoglobin light chains (α_2, α_{1F}, and α_{1S}; Fig. 6); transferrin (Fig. 25), Gc-globulin (Figs. 5 and 26; the left-most, more acidic two spots of the three labeled spots are Gc-1, and the right-hand, basic spot is Gc-2), α_2HS-glycoprotein (Figs. 3 and 21), and α_1-antitrypsin (Figs. 3 and 23). The Gc-globulin forms shown in Fig. 26 are interesting in that one common allele (form 2, panel C) consists of a single spot, and the other (form 1, panel B) consists of two major spots and a third minor one. Krueger *et*

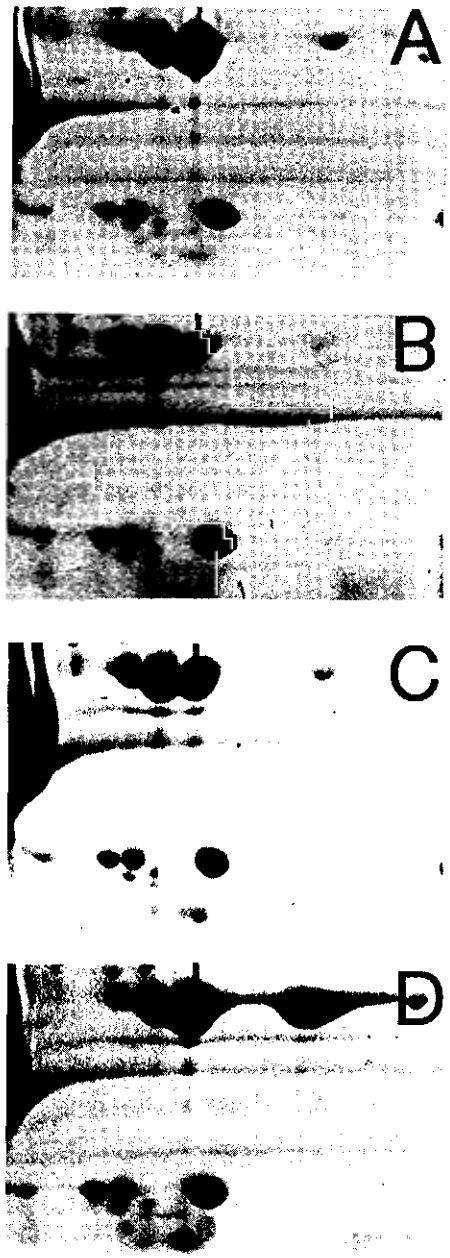

Fig. 25. Appearance of transferrin variants on two-dimensional patterns of serum proteins: (A) the normal form, (B) a homozygous acidic variant, (C) a heterozygote acidic variant, (D) a heterozygote basic variant. (Variant samples kindly provided by J. V. Neel.)

al. (1982) found a possible association of the Gc-2 spot with psoriasis, using two-dimensional gels, although they were not aware of the probable identity of their "abnormal" spot. An apoE lipoprotein polymorphism has been charac-

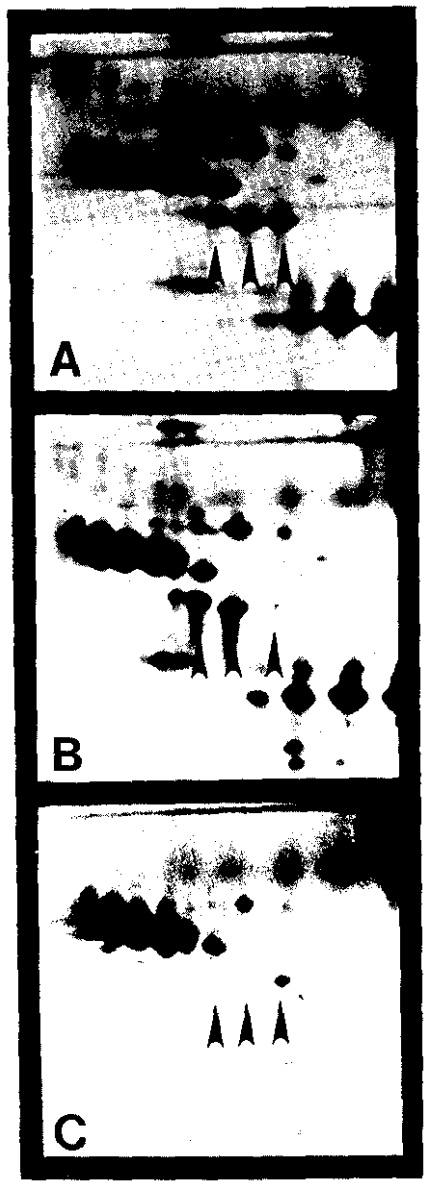

Fig. 26. Appearance of Gc-globulin alleles on two-dimensional gels (from Tracy *et al.*, 1982a). (A) Heterozygous Gc 1-2, (B) homozygous Gc-1, (C) homozygous Gc-2. (A) and (B) are plasma samples, and (C) is serum. Arrows point to the positions of the three principal Gc spots, the left-hand two of which constitute type 1, the right-hand (most basic) type 2.

terized by two groups using the two-dimensional approach (Borresen and Berg, 1981; Zannis *et al.*, 1981a). Both groups found a common allele present as an oblique row of three main components (the bottom being the most intense) at the

positions shown for apoE in Fig. 6 (apoE-III or ϵ-III) with two less common alleles represented by similar rows of spots displaced one charge toward the right (basic variant called apoE-II or ϵ-II) or one charge to the left (apoE-IV or ϵ-IV). Gene frequencies observed for the alleles II, III, and IV were 0.119 and 0.11, 0.770 and 0.72, and 0.111 and 0.17, respectively, as estimated by Borresen and Berg (1981) and Zannis *et al.* (1981a). Zannis *et al.* (1982b) have also demonstrated a possible genetic defect in apoA-I lipoprotein from patients with Tangier disease. In these patients, who have little or no circulating high-density lipoprotein, approximately one-half the recoverable plasma apoA-I is in the form of proapoA-I (Fig. 6) in contrast to less than 2% in normal subjects. Zannis *et al.* infer a possible genetic defect in the maturation of proapoA-I to apoA-I. Using two-dimensional gels, Mevag *et al.* (1981) have shown that an electrophoretic polymorphism of complement component C4 is due to charge differences in the C4 α chain (provisionally associated with protein PLS:9, Fig. 5). The C4 γ chain did not show charge variation, and the C4 β chain appeared too basic to test using the normal two-dimensional system. Bury and Roberts (1982) used two-dimensional electrophoresis to compare cystic fibrosis and normal plasmas, but were unable to find any differences that might confirm earlier controversial reports of a genetically determined cystic fibrosis serum protein (Wilson, 1979).

One particularly intriguing plasma protein polymorphism deserves further investigation. Unidentified protein PLS:31 is accompanied in presumed heterozygotes by a similar spot shifted approximately one charge more basic (to a position approximating that of spot No. 336 in Fig. 8). PLS:31 is unusual in that it appears to lack both carbohydrate and cysteine. Gene frequencies have been measured for the common (type 1) and basic (type 2) variant alleles yielding respective values of 0.927 and 0.073 (Rosenblum *et al.*, 1983; 62 individuals) or 0.910 and 0.090 (N. L. Anderson and J. V. Neel, unpublished; 268 individuals mostly unrelated). According to the frequencies already stated for the rarer allele, homozygous type 2 variants ought to occur at frequencies of 0.53% to 0.81%. None have yet been observed, however, either in the studies described previously or in serum samples examined at the Mayo Clinic (R. P. Tracy, unpublished results). PLS:31 may be the apoA-IV lipoprotein.

Other proteins in which rare variants have been observed using the two-dimensional approach include prealbumin and the fibrinogen α chain (Rosenblum *et al.*, 1983) and α_2HS-glycoprotein and the fibrinogen β chain (N. L. Anderson and J. V. Neel, unpublished).

C. Phylogenetic Comparisons

As yet, no systematic comparisons of animal sera have been conducted using the two-dimensional approach. Nevertheless, it seems worthwhile to describe the

basic ideas, if only to impel some investigator(s) to undertake the work. Two questions can be asked: (1) What are the relative charges of various proteins in a range of species, and how does charge variation change with evolutionary distance? (2) How different are the quantitative levels of various proteins in the species, and are there trends that correlate with evolutionary processes? Preliminary approaches to these questions using proteins synthesized by cell lines of 15 mammalian species ranging from bats and dolphins to man indicate that some proteins are highly conserved (mainly those with a structural role) while some are highly variable (showing no correlation of relative charge with species relatedness; N. L. Anderson, unpublished). It thus seems possible that if such a range exists in the plasma proteins, "molecular clocks" based on charge change substitutions having a variety of effective speeds could be found. It might also prove possible, using the wealth of information already available concerning plasma proteins, to correlate evolutionary charge variability with frequencies of variants in human populations and physicochemical properties on a protein-by-protein basis.

V. Immunoglobulins

From the viewpoint of two-dimensional mapping, immunoglobulins present special problems and special opportunities. In order to recognize and bind a seemingly unlimited number of foreign antigens, the serum immunoglobulins must themselves be able to take many subtly different molecular forms. The resulting heterogeneity, generated primarily by DNA-level rearrangements, is of such complexity as to overwhelm almost all physical (non-immunological) separation methods. Thus, the "spots" shown in the human plasma map (Figs. 1–6) to represent the serum immunoglobulins are not individual molecular species; rather they describe distributions of spots so numerous as to be unresolvable in normal human individuals using current techniques. However, this heterogeneity is not necessarily so daunting in inbred, laboratory-raised animals (N. L. Anderson, 1981), and provides interesting opportunities for monitoring clonal events in the B cell immune system.

A. Chain Identification

The IgG γ chains are relatively well separated from other proteins (except fibrinogen β chains), and were easily identified in the first plasma two-dimensional maps (N. L. Anderson and Anderson, 1977). The remaining heavy-chain types have been identified mainly through use of characterized human myelomas. Thorsrud *et al.* (1980) identified the α and μ heavy chains of IgA and IgM. This work was confirmed and extended by Latner *et al.* (1980a,b), who

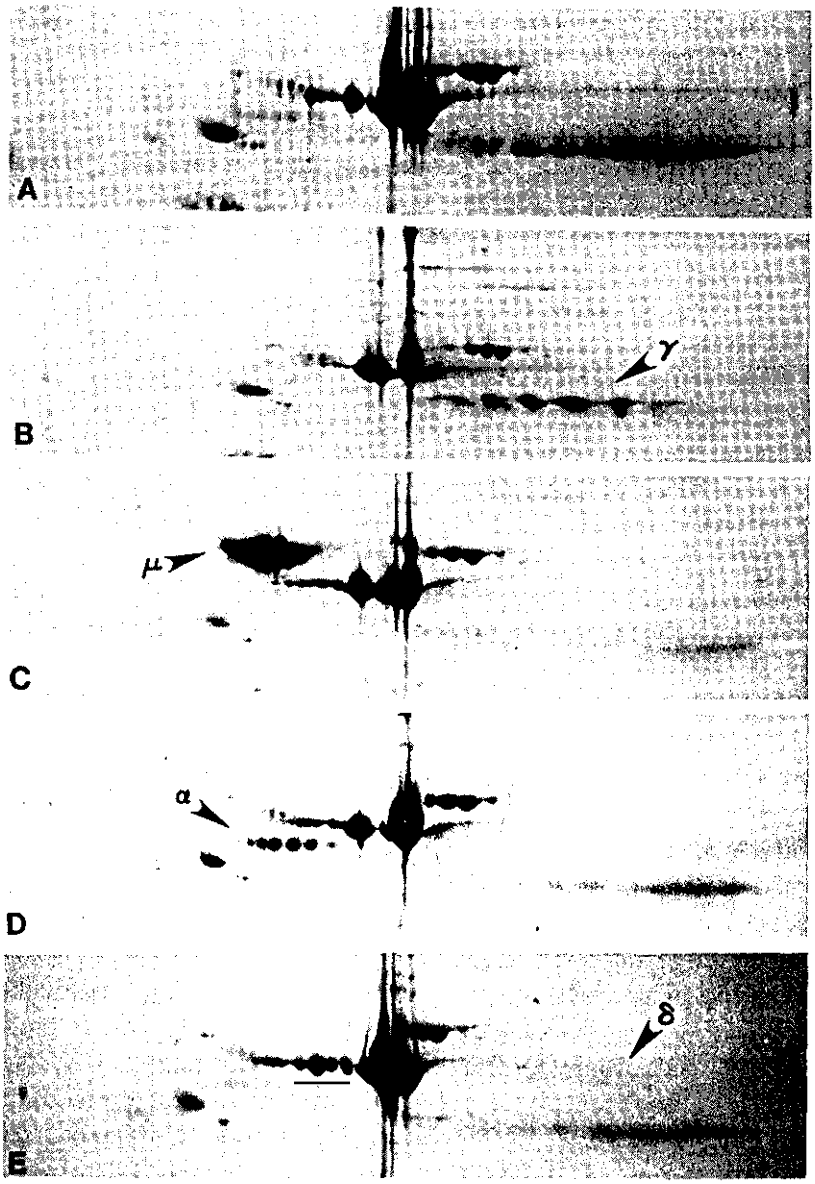

Fig. 27. Appearance of myeloma immunoglobulin heavy chains on two-dimensional gels (from Tracy *et al.*, 1982b). (A) normal serum, (B) IgG myeloma, (C) IgM myeloma, (D) IgA myeloma, (E) IgD myeloma.

identified α, μ, and IgD δ heavy chains. Tracy *et al.* (1982b) confirmed these identifications (Fig. 27). Jellum and Thorsrud (1982) added the position of the IgE ϵ heavy chain, and showed that the γ_3 chain formed a band at somewhat higher SDS molecular mass than γ_1, γ_2, or γ_4 chains. Tracy *et al.* (1982b) analyzed 45 γ chain myelomas, and found that, in general, γ_3 and γ_4 chains

appear at slightly higher apparent SDS molecular masses than γ_1 and γ_2, but that γ_4 and γ_2 are generally more acidic than γ_3 and γ_1. IgG γ_1 and γ_2 were well discriminated on the basis of p*I* alone using a p*I* value of 8.1; 17 of 18 γ_1 and 10 of 11 γ_2 myeloma proteins were correctly separated.

The κ and λ light chains are largely separated in the SDS gel system, even though their sizes are very similar (N. L. Anderson and Anderson, 1977). Tracy *et al.* (1982b) examined this separation as a means of distinguishing κ and λ myelomas, and found that use of the best single SDS molecular mass discriminant value (constant across the gel) yielded 80% correct assignment of κ chains and 73% correct assignment of λ chains.

Other molecular species associated with the classic immunoglobulin chains include the J-chain [found in IgA and IgM (Tracy *et al.*, 1982b) and stained on nitrocellulose transfers using appropriate antisera (N. L. Anderson *et al.*, 1982)], the secretory piece of IgA [observed in saliva (Giometti and Anderson, 1980) and in an IgA myeloma ("unknown" spots in Fig. 2 of Jellum and Thorsrud, 1982)], and an unidentified glycoprotein of about 50 kilodaltons (SDS) which is associated with IgM and may be a μ chain fragment (Tracy *et al.*, 1982b; N. L. Anderson, unpublished). The unidentified protein is referred to here as μ-s.

B. Protein A–Binding Fraction

Most immunoglobulin binds to staphylococcal protein A at neutral pH. Protein A–Sepharose can thus be used to isolate immunoglobulins from serum in one step, after which they can be eluted with SDS or concentrated urea. Pearson and Anderson (1980) devised a microcolumn approach using 10-μl protein A–Sepharose columns prepared in disposable plastic pipette tips that allowed the analysis of large numbers of immunoglobulin samples. A sample of human serum total Ig prepared by this method is shown in Fig. 28. The major serum Ig chains (κ, λ, γ, α, μ, J, and μ-s) are easily observed. The major contaminating components, aside from very small amounts of albumin, apoA-I lipoprotein, and α_1-antitrypsin, are probably as yet unidentified complement components.

The studies done so far reveal clearly that current gels cannot resolve the Ig light chains of normal human serum (N. L. Anderson, 1981a). It should be noted, however, that the distribution of light chain density is nonuniform, appearing to be bunched in both p*I* and SDS molecular mass. This bunching pattern is very similar in all normal human sera. Pathological samples, however, often show a tendency toward increased clonality (Jellum and Thorsrud, 1982; N. L. Anderson, unpublished) which may be indicative of severe stress to the immune system.

Total protein A-bound Ig of the BALB/c mouse has quite a different appearance (Fig. 29). All three major groups of Ig chains (μ, γ, and light) are more nearly resolved into spots, the light chains particularly so (N. L. Anderson,

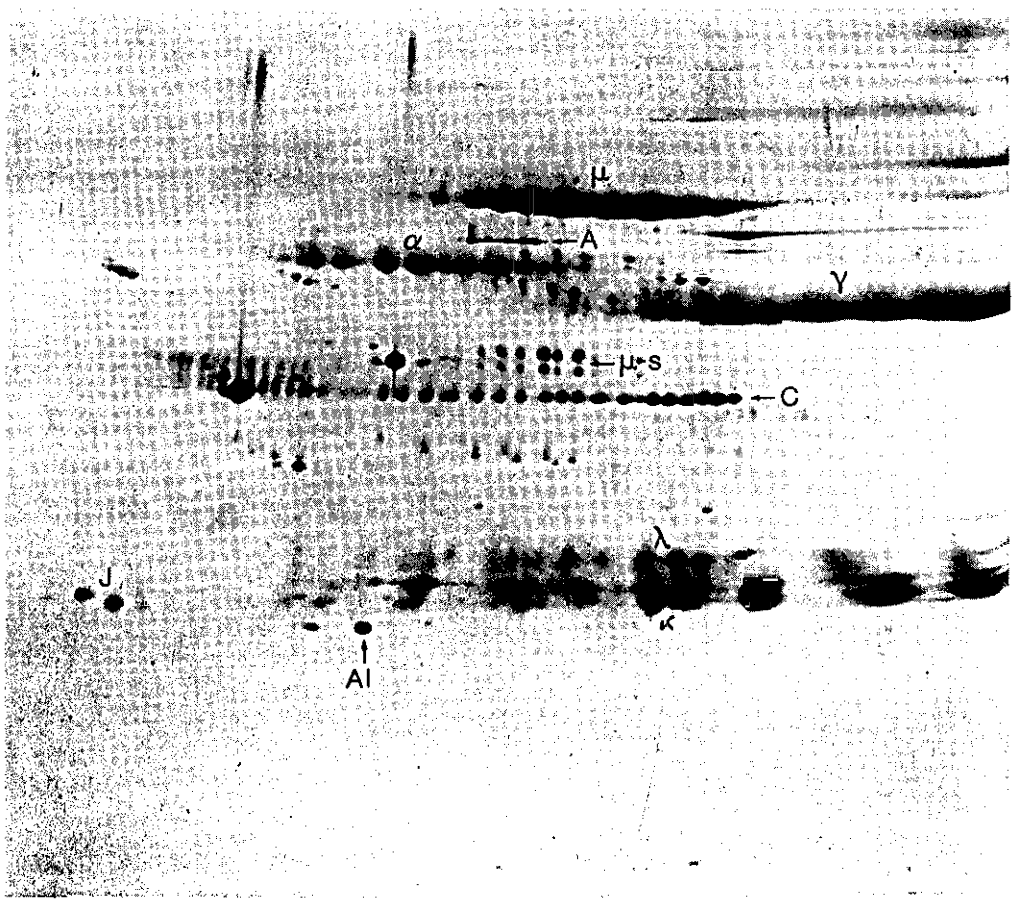

Fig. 28. Two-dimensional patterns of human serum protein A-binding material (primarily immunoglobulins). Greek letters label the respective Ig chains; J is the Ig J-chain, and μ-s is an IgM-associated protein. Albumin is A, apoA-I lipoprotein is AI, and C indicates the row of added creatine phosphokinase charge standards.

1981a). The upper level of the light chains (where the less-numerous λ chains are expected) appears as a region of distinct spots, while even the lower, more populated κ-chain region is distributed in much tighter clumps. Surprisingly, the distribution of light chain spots is quite similar in different individual mice (Fig. 30). Approximately 80% of the spots are shared (N. L. Anderson, 1981a), suggesting that many may be the products of unaltered germ-line light-chain genes. This conjecture is supported by the observation that light-chain patterns of C57 and BALB/c mice are not generally distinguishable, while light chains from a different, outbred species are both more numerous (about twice as many spots) and differently distributed (N. L. Anderson, 1981a). If even some of the resolved spots represent individual light-chain sequences, then two-dimensional electrophoresis, in conjunction with the sensitive silver stains, may make it possible

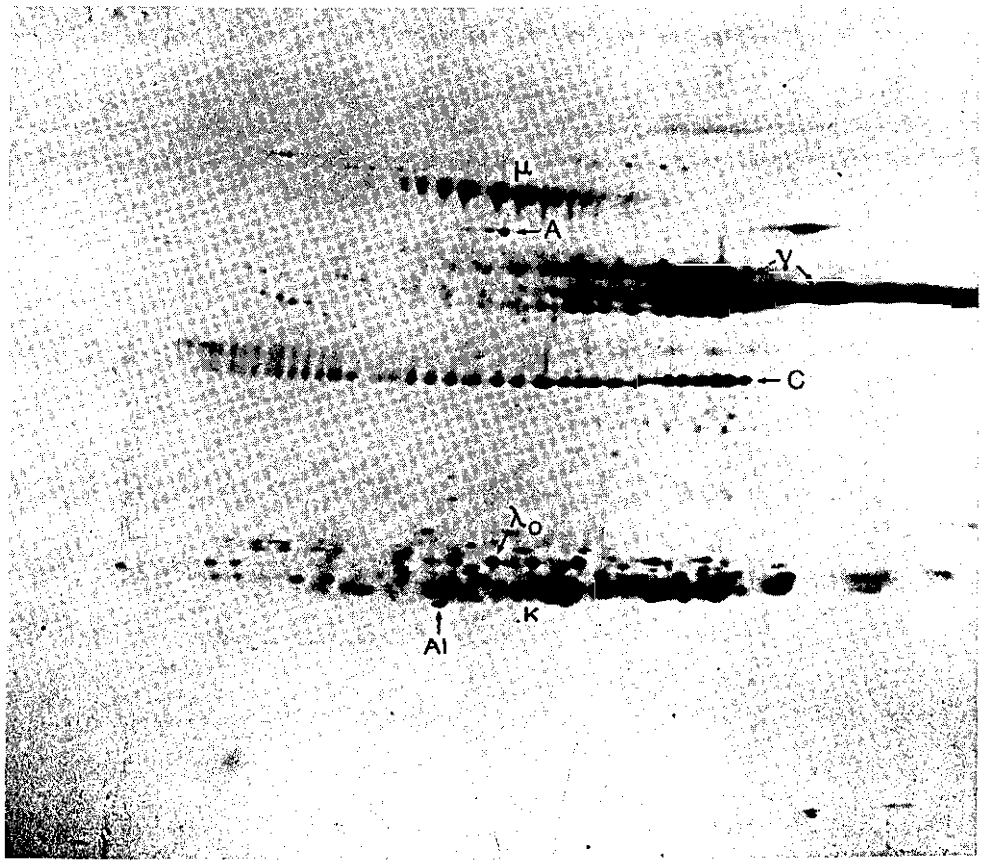

Fig. 29. Two-dimensional pattern of protein A-binding proteins from BALB/c mouse serum. Light chains are much more fully resolved than in Fig. 28. Candidate λ_o gene product is indicated by λ_o; other labels are as in Fig. 28.

to follow over time the fate of numerous B cell clones in a single animal. Given the steady increase in resolution that has occurred to date, it may ultimately be possible to perform such clonal surveys in man as well (N. G. Anderson and Anderson, 1981; N. L. Anderson and Anderson, 1981).

VI. The Future of Protein Mapping

The principal attraction of work with two-dimensional protein maps is the opportunity to see by inspection which protein components are present or absent in a sample, and which have been modified. The technique is clearly not an end in itself, but its development is made worthwhile by the very general importance of knowing the composition of living cells and body fluids. Although present maps are able to distill much of the information available concerning plasma

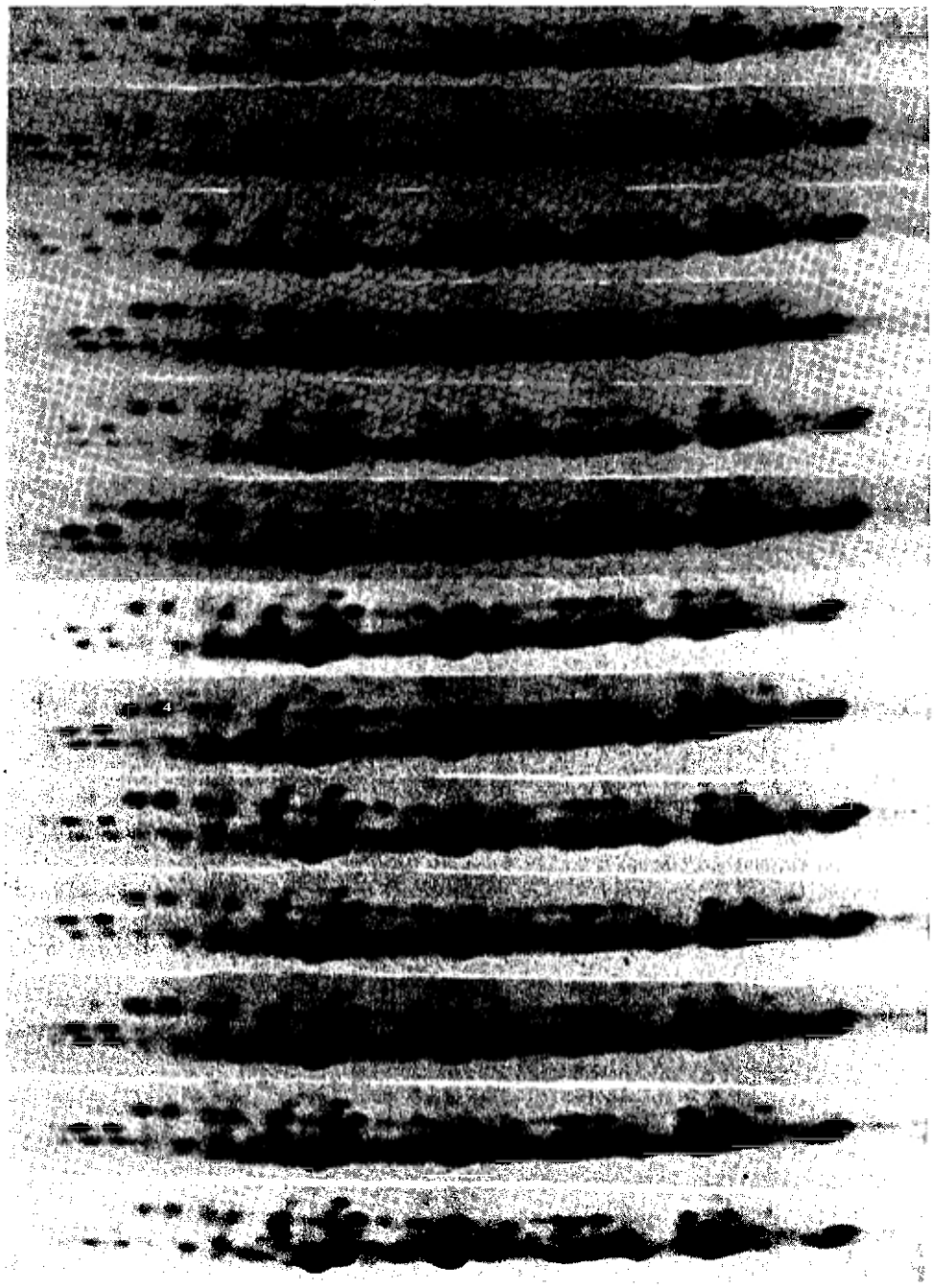

Fig. 30. Light chain regions from two-dimensional gels of protein A-bound Ig. Patterns from 13 individual mice are shown.

proteins into a unified, rememberable picture, it should strike readers as unsettling that large numbers of components remain unidentified. Complement and coagulation factors will account for some of these, but probably less than one-half. It is to be hoped that the use of two-dimensional mapping technologies will, by revealing details of a currently ignored background, encourage investigators to notice suggestive properties of unknown molecules in the course of ongoing research. The "discovery" of proteins may thereby cease to be a discontinuous process, and may become a simpler event more akin to recognition.

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