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CROSSED IMMUNOELECTROPHORESIS: QUALITATIVE AND QUANTITATIVE CONSIDERATIONS

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Invented 20 years ago, crossed immunoelectrophoresis (X-IEP) today is a technique of unusual power and myriad application. It combines very high resolution with exquisite specificity by alloying 2-dimensional electrophoresis with immunoprecipitation for symbiotic new potentialities. The consequent matchless quantitative/qualitative capabilities of X-IEP for analyzing antigens in complex mixtures, particularly by their idiomatic internal comparisons, are still not widely recognized. Because of this and the supposed complications of its use and interpretation, X-IEP is more rarely used than it should be. This essay discusses contemporary X-IEP with the particular aims of demonstrating that it is not difficult to use and of explaining with selected examples why it is peculiarly powerful for analyzing antigen mixtures like the body fluids, tissue and cell extracts, and microbial homogenates.

Key words: crossed immunoelectrophoresis: qualitative and quantitative aspects — crossed immunoelectrophoresis: clinical applications

INTRODUCTION

In 1960, Newton Ressler first described a primitive, rather impractical form of immunoelectrophoresis now known as crossed immunoelectrophoresis. Later improvements (Laurell, 1965; Clarke and Freeman, 1968; Kröll, 1968; Weeke, 1970; Svendsen and Axelsen, 1972; Axelsen and Kirkpatrick, 1973; Platt et al., 1973) made crossed immunoelectrophoresis (X-IEP) into a technique with a vast array of applications and potentialities. X-IEP analyses have ranged from complex cellular antigens and serum proteins to specific genetic polymorphisms of singular proteins. But these analyses have been done by relatively few laboratories, the capabilities of X-IEP apparently still not being widely realized or fully appreciated. This seems due largely to three broadly held misconceptions about X-IEP: first, that it is difficult to use and confusing to interpret; second, that it offers essentially

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the same kind of information as any high-resolution zone electrophoretic technique; third, that it provides analyses of mixtures of antigens no different than could be obtained by analyzing those antigens in mixture individually. We hope here to correct these misleading ideas. Consequently, this will be a selective review of the technique, main principles, and illustrative uses of contemporary X-IEP. For more general reviews see Axelsen et al. (1973b), Verbruggen (1975) and Owen and Smyth (1977).

PROCEDURES AND PRINCIPLES

Simplified procedure

Today, X-IEP is nearly as simple and economical as classic micro-immunoelectrophoresis. Here, for instance, is a brief account of the procedure we use (cf. Crowle, 1981; Emmett and Crowle, 1981).

A 0.4 mm thick, flat, and even layer of 1.4% agarose containing 1.0% dextran is cast between two 50 mm × 75 mm glass slides separated by small corner spacers. The top slide (not coated with 0.2% agarose) is drawn off of the gel attached to the other slide (pre-coated) after the gel has cooled and hardened. This 'sandwich' is then replaced by another consisting of the gel and another uncoated slide, also separated by spacers. These slides are offset so as not to cover a 15 mm × 75 mm zone of the gel. Next, approximately 0.8 ml of antiserum is injected between the slide and gel surfaces. The antiserum conforms to the covered gel area. That is, during a subsequent 30 min incubation at 37 ° C, antibodies from the antiserum diffuse into all parts of the gel except the 15 mm × 75 mm zone through which the antigen mixture to be analyzed will be given its first electrophoresis. After this incubation, the charging slide and excess antiserum are removed. The antiserum can be stored for one or two re-uses after appropriate concentration.

A 1 mm hole is punched in one end of the antibody-free gel and filled with 1 μl of the antigen mixture to be analyzed (e.g., serum). Then, it is electrophoresed for 45 min through the antibody-free zone (Fig. 1, top) and 2.5 h, at a right angle to the first direction, up into the antibody-charged zone (Fig. 1, bottom). There, the antibodies have remained essentially immobile throughout both electrophoretic runs due to the conditions of analyses (low endosmotic agarose, pH 8.6 buffer: cf. Laurell, 1965; Versey and Slater, 1973; Verbruggen, 1975). By the end of the second electrophoresis, the X-IEP pattern is well developed and can be examined preliminary by dark-field lighting. After 24 h more of standing at 4 °C, it is washed and stained for final analysis and/or photography.

The two procedures as described above that have greatly simplified this technique are sandwich casting and antibody infusion. The latter not only avoids difficult gel cuttings and castings typical of earlier techniques (e.g., Laurell, 1965; Clarke and Freeman, 1968), but also simplifies the placement and use of various antibodies or antigens in a gel and of various additives, like such surfactant solvents as Triton X-100 at concentrations that would prevent agarose gelling (Crowle, 1978).
Fig. 1. X-IEP development. In X-IEP, a mixture is first electrophoresed through antibody-free agarose (top), and then the antigens separated during first electrophoresis are electrophoresed up into gel charged with polyclonal antiserum against the antigens (bottom). Human serum antigens shown in the top panel were fixed and stained for this demonstration, because in X-IEP their presence is not evident until they are precipitated by antibodies during the second electrophoresis.
Interpretive principles

Basically, X-IEP owes its unique analytic powers to superb qualitative and quantitative resolution combined with definitive immunochemical identification (Weeke, 1973). Various kinds of zone electrophoresis identify macromolecules only by mobility. Therefore, there is no way of knowing in zonal analyses, without the supplementary tests, whether a single band, for instance, represents one or several overlapping proteins, or whether multiple bands represent shifting proteins or simply isomers of one protein. In 1953, Grabar and Williams greatly diminished many of these uncertainties by inventing immunoelectrophoresis and using specific immunoprecipitation for definitively identifying each macromolecule in agar gel zone electrophoresis. But, immunoelectrophoresis is not quantitative. Also, when it is used with complex mixtures of antigens, its immunoprecipitation pattern is too crowded for good resolution. X-IEP as developed by Laurell (1965) and finely exploited by such workers as Clarke and Freeman in a series of papers, by Weeke (1970) and by the Axelsen school (Axelsen et al., 1973b) overcame these shortcomings. In X-IEP profiles, antigens precipitate as high loops readily distinguished from each other even when electrophoretically identical, because they are antigenically different and, thus, precipitate independently. Moreover, the height/area of a loop is directly proportional to antigen quantity, and inversely to antibody concentration.

A classic example of erroneous analysis of human serum by zone electrophoresis that can be avoided by X-IEP illustrates the above principles (cf. Cline and Crowle, 1979). In fresh serum, the α and β lipoproteins are well separated. But, in stored and certain pathologic sera, a cathodic portion of the α migrates as a pre-β and is erroneously interpreted as such in zone electrophoresis where it is detected undiscriminatingly as a lipid. But, this shift is identified in X-IEP, because α and β lipoproteins are different antigenically; no error is made. Furthermore, the relative quantity of each can be accurately measured by the area circumscribed by α and β loops of precipitate, one without interference from the other but nevertheless in ratio to each other, to other serum antigens, and to a marker antigen added in known quantity to the analyzed serum.

These basic principles of immunochemical identification, discrimination, measurement, and, in particular, multiple ratio analyses are what make X-IEP uniquely powerful. An X-IEP pattern looks complicated to the novice, but regular users quickly become acquainted with individual antigens in a pattern. Qualitative or quantitative changes are readily apparent to them subjectively and can be documented, as necessary, quite objectively and reliably. At present, the great challenges presented by X-IEP are the identification of antigens in complex mixtures and discovery of their functions, fertile horizons even for something as familiar as human serum and essentially infinite in biology.
IDENTIFICATION AND CHARACTERIZATION OF ANTIGENS

The multivariate analytic capabilities of X-IEP have allowed the immunological dissection of complex systems of antigenic macromolecules not

| TABLE 1 |
|-----------------|------------------|
| Variety of cell antigens analyzed by X-IEP. | References |
| **Organism** | |
| Aleutian disease virus | Aasted (1980) |
| Candida albicans | Svendsen and Axelsen (1972); Axelsen et al. (1974); Axelsen et al. (1975); Greenfield and Jones (1981) |
| Coronaviruses | Schmidt and Kenny (1981) |
| Dermatophytes | Svejgaard and Christiansen (1979) |
| Fasciola hepatica | Hillyer and Cervoni (1978) |
| Herpes simplex viruses types 1 and 2 | Vestergaard and Bøg-Hansen (1975); Vestergaard and Grauballe (1975) |
| Micropolyspora faeni | Kurup et al. (1981) |
| Mycobacterium bovis | Closs et al. (1980); Harboe (1981) |
| Mycobacterium leprae | Closs et al. (1979) |
| Mycobacterium lepraeum | Closs et al. (1975) |
| Mycobacterium simiae | Thorel (1976) |
| Mycobacterium smegmatis | Kronvall et al. (1975) |
| Mycobacterium tuberculosis | Roberts et al. (1972); Wright and Roberts (1974); Kaplan and Chase (1980a,b) |
| Mycoplasma arginini | Alexander and Kenny (1980) |
| Mycoplasma pneumoniae | Schütten et al. (1980) |
| Neisseria meningitidis | Hoff and Høiby (1978a,b) |
| Nocardia species | Widebäck et al. (1980) |
| Pasteurella multocida | McKinney and Rimler (1981) |
| Plasmodium falciparum | Jepsen and Axelsen (1980); Jepsen and Andersen (1981) |
| Pseudomonas aeruginosa | Høiby (1975a,b; 1977a,b); Høiby et al. (1980) |
| Salmonella typhimurium | Kuusi et al. (1981) |
| Staphylococcus aureus | Espersen and Hedström (1981); Espersen and Schiøtz (1981); Kleppe (1981) |
| Staphylococcus epidermidis | Espersen et al. (1981) |
| Streptococcus salivarius | Weerkamp and McBride (1981) |
| Treponema pallidum | Pedersen et al. (1981) |
| Yersinia pestis | Głośnicka and Gruszkiewicz (1980) |
| **Mammalian cell antigens** | |
| Armadillo liver | Negassì et al. (1979) |
| Human liver | Greene et al. (1972) |
| Human lymphocytes | Wiik et al. (1979) |
| Human adenocarcinoma | Chakrabarty et al. (1981) |
| Human erythrocytes | Bjerrum and Lundahl (1973) |
TABLE 2
X-IEP analyses of body fluids.

| Human fluids          | References                                           |
|-----------------------|------------------------------------------------------|
| Serum, plasma         | Brogan et al. (1975); Laine and Hayem (1976)         |
| Sputum                | Bock (1973); Schmidt (1980)                          |
| Cerebrospinal fluid   | Eckersoll and Beeley (1980); Joneja et al. (1982)    |
| Parotid saliva        | Coombes et al. (1979)                                |
| Urine                 |                                                      |
| Mouse                 |                                                      |
| Serum                 | Quintero and Crowle (1979); Crowle and Miller (1981); Miller and Crowle (1982) |
| Rat                   |                                                      |
| Serum                 | Abd-El-Fattah et al. (1976); Scherer et al. (1977); Schade and Bürger (1979) |

Immunological comparisons between man and other mammals

| Armadillo — serum proteins | Negassi et al. (1979) |
| Primates — pregnancy-associated plasma proteins | Lin and Halbert (1978) |

a As X-IEP is most often used to analyze serum and plasma, the references are too numerous to list here.

directly possible with any other techniques. Unexpectedly, large numbers of antigenic constituents of many clinically important microorganisms, first detected by X-IEP, have been mapped, and, then, catalogued (Table 1). Already sophisticated analyses of the macromolecular components in human and animal body fluids done with classic immunoelectrophoresis are being considerably expanded and improved (Table 2). Such analyses are providing both qualitative and quantitative standard profiles necessary for identifying and studying disease-associated effects. These analyses have relied most on 3 modifications of basic X-IEP: X-IEP with an intermediate gel; tandem X-IEP; and utilization of monospecific antisera.

X-IEP with intermediate gel (Svendsen and Axelsen, 1972; Axelsen and Kirkpatrick, 1973)

Fig. 2 depicts the immunological comparison of mouse and human sera with intermediate gel X-IEP. Subsequent to their separation during the first electrophoresis, the mouse serum proteins have to pass through an area containing antiserum against human serum in second electrophoresis before entering that charged with anti-mouse serum. Both kinds of antiserum in the slide shown are applied, in sequence, without cutting or additional gel casting by the methods of diffusion described above (Crowle and Miller, 1981).
Fig. 2. Intermediate gel X-IEP as illustrated by a demonstration of the cross-reactivity of mouse and human serum antigens. Polyspecific antiserum to human serum was used in the intermediate area (H), and polyspecific antiserum to mouse serum in the upper area (M). Loops of precipitate formed by cross-reacting antigens extend down into the intermediate area, while non-cross-reacting antigens precipitate only in the upper area (e.g., loops indicated by arrows).

This figure, thus, illustrates a comprehensive and definite analysis of 2 very complex mixtures of antigen by an easy version of X-IEP. With an X-IEP map of human serum, Fig. 2 immediately and definitively can identify several major mouse serum antigens (cf. Crowle and Miller, 1981). Thus, many loops of precipitate extend into the intermediate gel, while some are entirely within this area. This indicates that there are cross-reactive antibodies in the antiserum against human serum that bind and precipitate homologous mouse serum proteins. It demonstrates that several proteins in mouse and human sera are antigenically closely akin.

Precipitating antibodies against specific cell antigens have been found with the intermediate gel technique in the sera of most patients infected with the organisms listed in Table 1. This technique, in addition, has been incisively utilized for taxonomic studies of Mycobacterium species (various authors), and comparisons of antigens from Nocardia and Mycobacterium (Widebäck et al., 1980). Used to study antigenic crossreactivity of Pseudomonas aeruginosa cell constituents with those of other bacterial species (Høiby, 1975a),
X-IEP has revealed a ‘common antigen’ for many of the bacteria studied (Sompolinsky et al., 1980).

**Tandem crossed immunoelectrophoresis (Krøll, 1968)**

A frequent variation of this technique, as originally described by Krøll, is to cut a second, reference antigen well slightly below the initial path of antigen migration from the main well containing the mixture of antigens being studied. In tandem X-IEP, antigens migrate in tandem from their respective origin wells. Since they also maintain their initial displacement during second electrophoresis, their closely juxtaposed loops of precipitins come into contact and interact according to Ouchterlony’s classic rules for antigen identifi-

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**Fig. 3. Methods for characterizing antigen precipitates in X-IEP.** Tandem X-IEP is shown in A and B. Human serum albumin (HSA) and bovine serum albumin (BSA) were electrophoresed in A and B, respectively, for 5 min in the first direction from the origin well before that well was charged with whole human serum. In A, the HSA loop of precipitate fuses with human serum albumin in the whole serum, while in B, a very faint loop of precipitate formed by BSA stops where it meets the anodic descending leg of the serum albumin producing a reaction of partial identity (spur formation) by the continued leg of the human serum albumin (open arrow). C and D depict precipitate identification by monospecific antibody blockade of an antigen. The antiserum, placed immediately anodic to the origin, is anti-transferrin and in D has eliminated the carbamylated transferrin loop (Ct, see C) and greatly altered and diminished the transferrin (Tr) loop of precipitate without affecting other loops of precipitate in the X-IEP pattern.
cation, i.e., showing identity, non-identity, or various grades of relatedness (Axelsen et al., 1973a; Bock and Axelsen, 1973). Fig. 3A and 3B, prepared to illustrate this, depict immunological comparison by tandem X-IEP of purified bovine serum albumin (BSA) and human serum albumin (HSA) with the proteins in a normal human serum X-IEP profile. The complete merging of the HSA precipitate with the tall, albumin peak demonstrates immunological identity, while the arrest of the BSA precipitate at the serum albumin loop together with the serum albumin continuing beneath this spur indicate partial identity. Obviously, the uninterrupted crossing of the precipitates of other serum proteins through the tall, albumin peak depict non-identity. This technique can compare single antigens to other antigens or a complex mixture of antigens, or two complex antigen mixtures with each other, such as normal and pathological sera (Kröll, 1968) or cellular antigens from various sources.

**Monospecific antiserum (Platt et al., 1973)**

Monospecific antiserum can be used in X-IEP to identify an antigen by selectively precipitating it with monospecific antiserum during electrophoresis in the first direction. This results in disappearance of the antigen's loop of precipitate in the second electrophoresis. To accomplish this, monospecific antiserum is allowed to diffuse into a narrow region of gel anodic to the antigen well and in line with the first dimensional movement of antigens from the well.

Monospecific antisera against a variety of serum proteins are commercially available. Many of these antisera contain various (human serum) antigens added by the manufacturer to absorb undesired antibodies. These will be precipitated by the polyspecific antiserum being used in X-IEP unless they are removed from the reaction area. This is done by preliminary electrophoresis either backward or downward relative to the direction of first dimension electrophoresis (Aguzzi et al., 1976; Cline and Crowle, 1979). Then, while antibodies from the monospecific antiserum remain where deposited, the extraneous antigens move into one of the electrode vessels and can be discarded before starting the first dimension using fresh electrolyte in that vessel. After this preliminary electrophoresis, X-IEP polyclonal antiserum charging, antigen application, and electrophoresis are performed as described in a previous section.

Figs. 3C and 3D show how a loop of precipitate can be identified by such a procedure. Monospecific antiserum against transferrin has precipitated some of the serum transferrin as a horizontal chevron and thereby decreased the vertical transferrin loop, which, in addition, merges with the horizontal chevron in a reaction of monospecific precipitate. The horizontal precipitation by a monospecific antiserum is usually sufficient to sequester all of the antigen for most serum antigens, since most are less concentrated than transferrin. For these, the X-IEP loop is simply eliminated. Note that this has happened, in Fig. 3D, for the added carbamylated transferrin.
The availability of numerous monospecific antisera against human serum proteins has made this procedure the best for first characterizing these proteins in X-IEP profiles (Cline and Crowle, 1979) and subsequently identifying them in the various human and animal body fluids, for example, human parotid saliva (Joneja et al., 1981) and mouse serum (Crowle and Miller, 1981).

**Preparation of monospecific antisera (Crowle et al., 1972)**

Monospecific antisera are powerful and increasingly popular analytic and preparatory reagents. The original procedures for their production against the various human serum antigens have been laborious and uncertain. They usually require complicated methods of antigen purification and risk denaturing the antigen during its purification. Immunodiffusion techniques, especially X-IEP because of its high resolution, have now made production of monospecific antisera certain and far easier. What took months or years, or may have been impossible a few years ago, now takes a few weeks. Historically, the use of preparatory X-IEP to make monospecific antisera will be one of the major technological advances in immunochemistry. But, as yet, few researchers know of or use this technique. Essentially, it provides a one-step positive purification of any antigen in a complex mixture in form and quantity sufficient to specifically immunize a rabbit.

The original mixture of antigens is fractionated by X-IEP using a polyclonal antiserum. The resulting X-IEP pattern is washed. The loop of precipitate, or part of the loop, representing the antigen of interest is cut from the pattern, homogenized, emulsified in Freund's incomplete adjuvant and injected into a previously unused rabbit. After the rabbit has been boosted once or twice more with the same kind of preparation it will begin to produce monospecific precipitins. This can be greatly amplified by boosting the animal with large quantities of the original mixture of antigens and bleeding the rabbit a few days later during its selective anamnestic response to the purified antigen.

Reasons why the minute amount of antigen obtained in an excised loop of precipitate from X-IEP suffices for this procedure are only fragmentarily understood. One must be that antigen-antibody complexes formed in slight antigen excess are powerful stimulators of antibody production (Laissue et al., 1971; Klaus, 1978, 1981). Whatever they are, their effects can be surprising. It is possible, for example, to identify by this procedure different antigens associated with anodic and cathodic subpopulations of human serum albumin by immunizing rabbits with anodic and cathodic legs of the albumin loop of precipitate in X-IEP (Crowle et al., 1972). Preparatory X-IEP has been utilized to produce many types of monospecific antisera including those to serum proteins (Crowle et al., 1972), influenza A virus (Hornsleth et al., 1980), varicella zoster virus (Hansen et al., 1981), herpes simplex virus type 2 antigen (Vestergaard, 1975), and *Fasciola hepatica*, a trematode parasite (Hillyer and Cervoni, 1978).
Enzymes

The extensive, detailed review by Owen and Smyth (1977) demonstrates the versatility of crossed immunoelectrophoresis in the study of enzymes. Table 3 illustrates the diversity of enzymes analyzed by X-IEP. Most studies involve the incubation with the substrate mixture and detection of enzymatic activity after electrophoresis and immunoprecipitation. Uriel (1963) has shown that antibody binding to an enzyme antigen and its precipitation does not inhibit most enzymes, even when an immunoelectrophoresis pattern has been washed and dried. Consequently, enzymes can be detected both immunologically and enzymatically, affording the capability of analyzing both enzymatically active and inactive forms. Furthermore, X-IEP can detect enzymes, such as cholinesterase and arylesterase (Brogren and Bøgh-Hansen, 1975), which do not form immunoprecipitates sufficiently intense to be seen with a stain for proteins. Other analyses include effects of inhibitors, substrate specificity, and enzyme-substrate complex formation or enzyme degradation as revealed by electrophoretic shifts.

QUALITATIVE ALTERATIONS

Many proteins exhibit qualitative variations under normal or pathological conditions. Crossed immunoelectrophoresis, with its resolution of proteins by electrophoretic mobility and immunological specificity, is uniquely able to detect these differences, whether they result in subtle changes in precipitate shape or in substantial shifts of electrophoretic mobility. For example, X-IEP with agarose has been used to demonstrate the partial degradation of human serum proteins after treatment with trypsin (Bjerrum and Bøgh-Hansen, 1975). The patterns made with trypsinized serum show new loops and lines of precipitate in addition to alterations of the original loops (e.g.,

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**TABLE 3**

Variety of enzymes analyzed by X-IEP.

| Enzyme                        | Description               |
|-------------------------------|---------------------------|
| N-acetyl-β-glucosaminidase    |                           |
| Acid and alkaline phosphatases|                           |
| Aminopeptidases:              |                           |
| alanine, leucine, glytamyl    |                           |
| α- and β-amylases             |                           |
| Arylesterase                  |                           |
| Arylsulfatase                 |                           |
| ATPase                        |                           |
| Ceruloplasmin                 |                           |
| Cholinesterase                |                           |
| Chymotrypsin                  |                           |
| Dehydrogenases:              |                           |
| alcohol, lactate, NADH        |                           |
| diphtheria toxin              |                           |
| elastase                      |                           |
| enolase                       |                           |
| β-glucuronidase               |                           |
| hexokinase                    |                           |
| non-specific esterase         |                           |
| NTPase/NDPase                 |                           |
| peroxidase                    |                           |
| phosphodiesterase             |                           |
| plasmin                       |                           |
| subtilopeptidase              |                           |
| trypsin                       |                           |

*From Owen and Smyth (1977).*
new shoulders, and skewing). More importantly, most of these new precipitates exhibit interactions of either complete or partial immunological identity (see section on tandem crossed immunoelectrophoresis) with their parental loops, something protein bands in polyacrylamide electrophoresis cannot do. Complexes between $\alpha_1$-antitrypsin and IgA, or fibrinogen (Laurell and Thulin, 1975), and between enzymes and their substrates (Owen and Smyth, 1977) also cause electrophoretic shifts of either part or the entire precipitate.

Various dyes have been known for many years to bind to serum ‘albumin’ (cf., Muckle, personal communication, 1976, 1978). X-IEP clearly shows that one of these sometimes used as a marker during zone electrophoresis, Evans blue dye, actually binds more to the $\alpha_1$-lipoprotein than to albumin (Crowle, 1973; Cline and Crowle, 1979). This dye not only binds to but also changes the mobility of albumin, $\alpha_1$-lipoprotein, $\alpha_1$-antichymotrypsin, hemo- pexin, and $\beta$-lipoprotein, according to the concentrations of it added to serum (Cline and Crowle, 1979). These changes, readily seen in X-IEP, would have been difficult or impossible to detect by zone electrophoretic analyses.

In addition to changes caused by interactions with other proteins or chemicals, many enzymes and serum proteins exhibit genetically determined electrophoretic polymorphism. Many of these differences in molecular size are too subtle to be evident in the non-sieving agarose gel of conventional X-IEP. Consequently, a number of investigators have used polyacrylamide or starch to improve protein resolution during the initial separation, and then electrophoresed the finely separated proteins from these media into antiserum-charged agarose for the second electrophoresis of X-IEP (Verbruggen, 1975; Owen and Smyth, 1977). Others have used polyacrylamide-agarose mixtures for the first direction gel, e.g., for studying $\alpha_1$-antitrypsin polymorphism (Laurell and Persson, 1973). Thus, while conventional X-IEP in agarose is itself quite powerful for resolving different antigens in mixtures, modest changes to alternative media or methods for first-dimension electrophoresis can increase its resolution still more to readily detect small electrophoretic differences among subpopulations of a single antigen.

Some qualitative differences can be detected among antigens in X-IEP that are not electrophoretic but compositional (e.g., the presence of carbohydrates). These can be seen by adding selective reactants to the agarose gel. Bøg-Hansen et al. (1975) introduced ‘immunoaffinoelectrophoresis’ as an addition of the lectin concanavalin A (con A) in either the first dimension gel (Bøg-Hansen and Takeo, 1980) or an intermediate gel (Bøg-Hansen and Brogren, 1975; Bøg-Hansen et al., 1978) to detect different serum glycoproteins and glycoprotein allergens in cow dander. Crossed (immuno)affinoelectrophoresis has also demonstrated alterations in lentil lectin binding by maternal serum $\alpha$-fetoprotein during gestation (Kerchaert et al., 1980).

In qualitative analyses, one of the more important applications of X-IEP is to directly detect pathogenic alterations of serum proteins as illustrated in Fig. 4. Fig. 4A is the serum profile from a lymphoma patient. This individual’s pat-
tern exhibits a number of quantitative abnormalities to be discussed in the next section, while the most noticeable qualitative change is the appearance of an anodal extension of the \( \alpha_1 \)-lipoprotein loop (compare with normal serum, Fig. 4C). This electrophoretic shift in the \( \alpha_1 \)-lipoprotein population could have clinical meaning for the management of this patient. Such a change is seen to appear and reverse during anaphylaxis and recovery in mice (Quintero and Crowle, 1979). The value of X-IEP in definitively showing changes such as this should be more widely recognized among clinicians so that the medical correlates of the changes can be understood and exploited.

The diagnostic value of qualitative changes in X-IEP profiles is readily evident for changes that are understood. For example, that of the patient in Fig. 4B clearly depicts a qualitative abnormality in IgG indicating monoclonal gammopathy. This patient’s serum profile is shown here because it
illustrates the power of specific analysis in X-IEP: zone electrophoresis of his gamma globulins had been interpreted as 'normal'. X-IEP clearly shows that while globulins in the gamma region could be considered collectively to be in normal concentration, their individual concentrations and the quality of the IgG is decidedly abnormal and simply totals to the 'normal' value undiscriminately detected by zone electrophoresis. Instances like this indicate that X-IEP must replace various kinds of zone electrophoresis in analyses of patients' sera, for it is obvious that zone electrophoresis data can be dangerously deceptive.

**QUANTITATIVE ALTERATIONS**

Perhaps the most important application of crossed immunoelectrophoresis is its precise, consistent, multifactorial quantitation of the pathological changes of proteins. Thus far, these analyses have been largely restricted to serum proteins, but their principles can be applied to many protein mixtures.

In their classic study of serum by X-IEP, Clarke and Freeman (1968) first described these quantitative capabilities. They established the direct relationship of precipitation peak areas to the concentrations of serum proteins. This was an important distinction from a similar relationship between peak heights and antigen concentration in rocket immunoelectrophoresis and relates to antigen diffusion during the first electrophoresis of X-IEP, but absent in rocket immunoelectrophoresis.

Loop areas in X-IEP are measured in several different ways including planimetry (Clarke and Freeman, 1968), cutting and weighing drawings on paper of precipitate outlines (Weeke, 1973), and considering the precipitates to be triangles and calculating the areas either as height × half-width (Firestone and Aronson, 1969) or as 0.5 × base × height (Versey and Slater, 1973). All of these methods are usefully accurate and roughly comparable.

In the same report, Clarke and Freeman made another major technical contribution to the precision of antigen quantitations by X-IEP. This was to add a quantitative reference marker. They originally used acetylated albumin; carbamylated transferrin (Weeke, 1970) now being preferred because of its migration near the albumin while acetylated albumin tends to migrate too anodically and run off a slide. This served 2 important functions. First, the marker indicated sample volume so that variations in sample application could be compensated for in estimating quantities of antigens. Second, the marker was a standard for comparisons by ratio of antigens in different sera and thus of different X-IEP profiles. Thus, a difference between ratios of any antigen to the marker for one serum compared with another serum was real, not technical.

The resolving power of X-IEP allows for the separation and detection of over 30 serum proteins with polyspecific antiserum (Emmett and Crowle, 1981). Consequently, X-IEP is capable of quantitating and comparing the concentrations of many proteins simultaneously, and, as a result, document-
| Antigen | IgG | C3 | Tr | IgA | Hpx | Hpt | LoL | α1-Lp | α1-AI |
|---------|-----|----|----|-----|-----|-----|-----|-------|-------|
| 5 successive analyses, same female A/J serum | 1.97 | 1.54 | 1.51 | 2.54 | 1.21 | 1.02 | 1.14 | 0.97 | 0.58 |
| Mean | 1.50 | 0.97 | 0.95 | 0.81 | 0.85 | 0.05 | 0.06 | 0.08 | 0.09 |
| S.E.M. | 0.08 | 0.09 | 0.12 | 0.12 | 0.08 | 0.05 | 0.05 | 0.12 | 0.04 |
| S.E.M./mean | 0.04 | 0.06 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.08 | 0.04 |
| 6 successive analyses, each different female A/J serum | 1.50 | 1.52 | 0.97 | 0.97 | 0.97 | 0.08 | 3.11 | 1.99 | 0.15 |
| Mean | 0.94 | 1.05 | 0.05 | 0.07 | 0.08 | 0.06 | 0.05 | 0.31 | 0.08 |
| S.E.M. | 0.04 | 0.06 | 0.05 | 0.07 | 0.05 | 0.05 | 0.10 | 0.03 | 0.04 |
| S.E.M./mean | 0.05 | 0.06 | 0.05 | 0.07 | 0.05 | 0.05 | 0.10 | 0.03 | 0.04 |
| 3 successive analyses, each a different male A/J serum | 0.90 | 1.04 | 1.41 | 1.53 | 0.54 | 0.05 | 1.67 | 1.29 | 2.92 |
| Mean | 0.90 | 1.04 | 1.41 | 1.53 | 0.54 | 0.05 | 1.67 | 1.29 | 2.92 |
| S.E.M. | 0.18 | 0.06 | 0.12 | 0.12 | 0.06 | 0.06 | 0.04 | 0.01 | 0.04 |
| S.E.M./mean | 0.20 | 0.07 | 0.08 | 0.08 | 0.08 | 0.06 | 0.04 | 0.008 | 0.008 |

a From Crowle and Miller (1981) with permission.

b For each X-IEP pattern the area of every loop was divided by the area of the α2-M loop in the same pattern to obtain the ratio for that loop.

c Antiserum for this series of analyses different batch from that used for the other two series in this table.
ing the changes of these proteins under pathological conditions. These multifactorial analyses have been used to examine the serum abnormalities associated with such diseases as tuberculosis and sarcoidosis (Clarke et al., 1970b), multiple sclerosis (Clarke et al., 1970c), and rheumatoid arthritis (Clarke et al., 1970a). As a result of its versatility and sensitivity, X-IEP has been able to detect serum protein differences due to age and sex (Clarke and Freeman, 1968; Weeke and Krasilnikoff, 1970; Crowle and Miller, 1981; Miller and Crowle, 1982), and to differentiate one person’s pattern from another (Kashimura et al., 1979). Furthermore, the precision and consistency of ratios in X-IEP permits reliable comparisons between small populations of individuals, in spite of the normal protein variations. As shown in Table 4, from a study of mouse sera (Crowle and Miller, 1981), X-IEP ratios are consistent enough for statistically significant comparisons to be made among small population samples. Using this capability, we have recently been able to discern several statistically significant serum protein differences between populations of tuberculosis (TB), adult respiratory distress syndrome (ARDS) and cystic fibrosis (CF) patients (ranging from 10–16 individuals) and a normal population of 13 subjects (Emmett et al., 1982).

Even though X-IEP has been shown to be a versatile and reliable quantitative tool, and despite evidence that zone electrophoresis by comparison is inferior, even deceptive (see above), X-IEP has attracted little use in the clinical laboratory. One reason seems to be its complexity compared with zone electrophoresis or with immunologic techniques that quantitate one antigen at a time. The typical X-IEP serum pattern with its approximately 30 loops of precipitate nevertheless is easy to reproduce and, for someone with only moderate experience with it, also easy to read. It forms by the same immunological principles as other immunodiffusion tests. X-IEP patterns are essentially mixtures of many rocket immunoelectrophoretic tests in a single pattern—each precipitate being a rocket for a particular protein.

But, there is one very important difference between X-IEP and monospecific tests. As a directly comparative analysis of multiple antigens with each other as well as with an added standard, and both qualitative and quantitative, X-IEP offers a profile of integrated physiological and pathological information impractical and impossible to obtain by single-analyses techniques. This is illustrated in Figs. 4C and 4D. These patterns compare normal and adult respiratory distress syndrome (ARDS) serum proteins in general, and their α₁-antitrypsin levels specifically. Note that these patterns show equivalent or ‘normal’ α₁-antitrypsin concentrations in both sera, as would a monospecific test for α₁-antitrypsin. But, since X-IEP also displays antigen relative quantities by ratio to each other and the standard marker (see above), it is obvious that the ‘normal’ ARDS α₁-antitrypsin concentration actually appears to be very high by ratio to most of the other ARDS serum proteins which are substantially depressed due to extensive losses into the lung (Petty, 1978). Therefore, the ARDS level of α₁-antitrypsin actually represents elevated production.
Figs. 4A and 4B provide further support for the clinical importance of viewing the whole serum profile as already discussed. In addition to the qualitative abnormalities already mentioned, both patients exhibit numerous quantitative alterations. Of note, are the decreases of albumin, and the polyclonal increase of IgA in one pattern (lymphoma), contrasting the apparent absence of IgA in the other serum (plasmocytosis).

X-IEP is a very powerful immunochemical technique for analyzing complex mixtures of antigen. We believe that it is being under-utilized, especially in medicine, largely because its unique capabilities still are not widely understood, but also because it appears to be too complex to use and interpret. We have attempted here to abate both of these impediments by explaining with illustrations its most important proficiencies and briefly describing current simplified ways in which it can be used and interpreted.

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