The use of electroimmunoassay for determining specific proteins as a supplement to agarose gel electrophoresis

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Electrophoretic analysis is the standard method of screening for abnormalities of the plasma proteins. To be acceptable, supportive media should have negligible adsorption and little interaction with protein, such as agarose and cellulose acetate. For high quality patterns the plasma proteins should separate over more than 5 cm within one hour, without the temperature of the supporting medium rising above 30°. The buffer should be slightly alkaline, and the addition of calcium (2 mmol/l) improves the resolution of the three major proteins in the β-zone by slowing the β-lipoproteins and the third factor of complement (C3), each to a different extent, whereas the mobility of transferrin is unaffected.

There are two principal methods of interpreting the patterns obtained. One is to relate each major fraction to the total protein after scanning or eluting the strips and to describe the plasma protein composition in terms of the values found for albumin, α₁, α₂, β, and γ-globulins. Experience of the changes in the electrophoretic fractions in various diseases, gained over many years, is an additional requirement for interpretation of the laboratory data.

The other method aims at a more profound analysis, and includes, in addition to electrophoresis, the specific analysis of a limited number of individual proteins to determine the contribution of these proteins to the groups separated by electrophoresis. This detects changes in the homeostasis of individual proteins, which are of pathophysiological interest since the metabolic regulation varies from protein to protein. Thirteen major proteins constitute more than 95% of the total plasma protein (fig 1). An analysis of the contribution made by these proteins to the different electrophoretic zones has been published (Laurell, 1972).

Comparison of the electrophoretic pattern with the results of analysis of the major proteins will also indicate whether other minor components, not normally recognizable in the electrophoretic pattern, are increased in a given disease.

It is evident that analysis of all 13 proteins will give more information than measurement of five or six heterogeneous electrophoretic fractions. However, it is by no means certain that the determination of the 13 most abundant proteins is of greater clinical value than that of some 20 other proteins which occur in plasma in such low concentration as not to contribute significantly to the electrophoretic pattern. Everyone who is responsible for the interpretation of electrophoretic patterns must learn to think in terms of specific proteins rather than electrophoretic fractions and to realize how variations in the concentration of different proteins influence the patterns.

It is debatable how many and which of the specific proteins should be examined to supplement the

Fig 1  Electrophoretic distribution of 13 major plasma proteins (from Laurell, 1973).
Electrophoretic strip in a given patient. The answer depends on the clinical problem, the laboratory facilities available, and the cost.

The determination of specific proteins usually requires immunochemical techniques, namely radial immunodiffusion (RID), electroimmunoassay, or automated immunoprecipitation (AIP). All three are sufficiently precise and sensitive for clinical requirements. Automated immunoprecipitation is the quickest and the most expensive, except for large-scale analysis, and needs the highest quality of antisera, whereas RID is the slowest, cheapest and simplest for the less experienced, although it is tedious for examining a large number of specimens. Electroimmunoassay occupies an intermediate position in respect of time and costs, and the antiserum used need not be so very specific; this technique has recently been described in detail (Axelsen, Krøll, Weke, 1973), and requires only a brief comment on the analytical principle. The protein antigen is carried by an electric field into a gel containing antibodies, the pH and electroendosmosis being such as to allow little or no migration of the antibodies to the cathode when the antigen migrates to the anode, or vice versa. Antigen-antibody complexes are formed at the front of the migrating antigen. When the antigen combines with an antibody its charge decreases and the complex therefore migrates more slowly behind the antigen front.

The solubility of the antigen, its molecular size, and the number of its antibody-combining sites determine the degree of antibody saturation at which the complexes will start to precipitate. The optimal ratio of antigen to antibody for precipitation in an electric field is different from that in free solution. Large antigens are precipitated in a zone of large antigen excess, while smaller antigens migrate until they approach an antigen-antibody ratio similar to that for precipitation in free solution. However, in rocket electrophoresis precipitation always occurs with a relatively higher ratio of antigen to antibody than is required for precipitation of the same system in solution. A large excess of antigen produces rockets with a more intense inner contour than does antigen near the optimal ratio for solutions. The fact that the antigen is only partially saturated with antibodies may be utilized for second stage 'sandwich' staining of the precipitates with isotopically labelled antibodies; this provides a sensitivity down to 1 μg per litre as compared with 10 mg per litre for standard electroimmunoassay, the latter being sufficient for the determination of most plasma proteins. A basic prerequisite for successful application of the method is a difference in charge between antigen and antibody. Antigen size is another variable that must be taken into account; if the size of the antigen is small compared with that of the antibody, the latter will substantially influence the mobility of the complex, and in extreme cases may reverse the direction of migration of the antigen. In general this limits the useful size range of antigens from 40 000 to 4 000 000, but small molecules such as α-globulin have also been successfully measured. The antiserum consumption in the electroimmunoassay is about 1 ml per 100 analyses and the method is more economical for batch analysis because it requires a series of four standards. Cooled electrophoresis equipment is recommended since it allows higher voltage (10-15 V per cm) with completion of the analysis within three to six hours, but less expensive equipment may be used if lower voltages and hence longer times are acceptable; a run with 0.05 M barbital buffer at about 5 V per cm overnight is then usually sufficient.

The decision which of the 13 proteins to determine depends on the purpose of the analysis. Neither scanning of strips nor analysis of specific proteins are required to recognize myeloma proteins, high or low polyclonal IgG, hypoalbuminaemia, signs of inflammatory response, or cirrhotic or nephrotic patterns. These and more are recognized at a glance. In fact, for general screening the gel pattern is sufficient. However, this gives only a crude assessment, and to assess the degree of abnormality or to study minor changes, quantitative data are necessary. Acquisition of such data for a number of proteins is not justified unless the examiner is experienced in the interpretation of their significance. Clinicians are unlikely to be able to interpret these data because they are unaware of the technical difficulties and do not have the advantage of being able to compare many patterns simultaneously.

Most clinicians rely on the radiologist to interpret their x-ray films, but he requires a clear statement of the clinical problem before deciding how to examine the patient and giving his report. Exactly the same procedure should be adopted for analyses of plasma proteins if the maximum of information is to be obtained. Interpretation of laboratory data in pathophysiological terms is usually impracticable in the absence of adequate clinical information. The cost of the clinician's time spent in providing such information on the request form is very much less than the cost of the investigation.

The clinician should state clearly the diagnostic or therapeutic problem. He should also give such information as length of history, body temperature, and date of trauma or operation etc, otherwise it is not possible for the clinical biochemist to decide whether or not, for example, an apparent inflammatory response (including the immunoglobulin
pattern) fits the case history. The presence of proteinuria or gastrointestinal protein loss or the administration of blood or blood substitutes, of cytotoxic agents, or of steroids should also be stated.

The report form should contain the normal ranges of the most commonly analysed of the 13 proteins which constitute more than 95% of the total plasma proteins (fig 1); \( \alpha \)- and \( \beta \)-lipoproteins and transferrin need not be included because simple inspection of the gel pattern is sufficient to detect any gross abnormality. The \( \alpha_2 \)-macroglobulin level has, as yet, little clinical significance in spite of its important biological functions. Haemopexin and orosomucoid stain less well than most proteins and are therefore not easily recognized in the patterns. Albumin is routinely analysed separately, usually with bromcresol green (BCG), but examination of the intensity of staining of the albumin zone will sometimes reveal probable discrepancies between the BCG values and the intensity of the electrophoretic band, especially in patients with hypoalbuminaemia. For this reason an immunochemical estimation is needed as a reference method and this, because of its sensitivity, is also useful for the analysis of cerebrospinal fluid and urine. This problem of accuracy is evident from fig 2 which compares BCG values and immunochemical albumin values. The material has been selected from the routine laboratory over a year to obtain a large series of hypoalbuminaemic patients. The spread is remarkable in the low range, where the dye-binding capacity of albumin varies markedly in relation to the number of antibody-combining sites. Plasma from patients with chronic renal or intestinal protein loss takes up a relatively larger amount of the dye per albumin content as measured by immunoprecipitation, and this does not develop until after weeks of continuous albumin loss. The chemical background is still obscure.

As standard indicators of the inflammatory response and related reactions we have chosen fibrinogen, haptoglobins, orosomucoid, and \( \alpha_1 \)-antitrypsin. This may appear overambitious as they usually vary in a similar fashion during infections, in aseptic necrosis, and after trauma (for references see Laurell, 1972), but each has its own value in particular cases.

Increased fibrinogen is a sensitive indicator of inflammation and more sensitive than most others, especially in chronic diseases affecting the connective tissues such as rheumatoid arthritis. It may be unreliable in acute and subacute inflammation if accompanied by increased fibrinolysis. On the other hand, slightly increased values are often found without coexisting signs of inflammation in diseases with increased capillary permeability. Fibrinogen is the plasma protein that varies most closely with the erythrocyte sedimentation rate.

Haptoglobins and orosomucoid are also reliable indicators of an inflammatory response and give few, if any, false positive results. They usually increase in parallel and their synthesis appears to be controlled by a common regulator. However, considered individually, haptoglobin is less reliable than orosomucoid because its level may be normal or even low as a result of increased catabolism due to sepsis, or to decreased hepatosplenomegaly or bone marrow blood flow; furthermore low levels occur in any disorder with concomitant intravascular haemolysis. Any discrepancy between the levels of orosomucoid and haptoglobin may thus be of diagnostic significance and, in our opinion, both should be estimated far more often. Their levels reflect not only the presence of inflammation but also its intensity, in acute as well as in chronic conditions. Usually, failure of the haptoglobin level to increase at the same rate as orosomucoid indicates a complication. A slight increase of orosomucoid alone may be due to slightly reduced glomerular filtration or mild intestinal inflammation.

Alphai-antitrypsin \((\alpha_1 \text{-AT})\), which is also an acute phase reactant, is of special interest in liver disease. An absolute or relative increase in \( \alpha_1 \)-AT occurs, for example, in hepatitis and cirrhosis and contrasts with the normal orosomucoid and haptoglobin levels in these conditions. However, such a change is only suggestive of liver disease if the patient is neither pregnant nor taking oestrogens. In the latter cases caeruloplasmin also is raised.

As warning signs of an inflammatory process,
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A  Slight (S) and intense (I) inflammatory response, eg, trauma, infection, or aseptic necrosis.

B  Inflammatory response combined with increased haptoglobin catabolism, eg, septic infection, cancer with liver or bone marrow metastases. The broken line indicates the value of haptoglobin to be expected in the absence of increased catabolism.

C  Slight (S) and intense (I) inflammatory response in subacute to chronic disease affecting the connective tissues, eg, chronic rheumatoid arthritis.

D  Inflammatory response combined with fibrinolysis in a heterozygote for α1-antitrypsin deficiency. The broken line indicates values to be expected with inflammation in the absence of fibrinolysis in an otherwise normal subject.

E  Glomerular protein loss in the nephrotic syndrome (broken line = slight loss)

F  Cirrhosis due to hepatitis B virus, inactive (CI) and active (CA).

G  Late pregnancy (continuous line) and oestrogen-containing oral contraceptive (broken line).

Fig 3  Variations of the acute phase reactants. The boxes indicate the normal range.
acute phase reactants fail only on the first day, when C-reactive protein and antichymotrypsin are already increased.

With the few exceptions mentioned, orosomucoid seems to be the most reliable and the one best correlated with the extent and intensity of an inflammatory reaction. If the concentrations of these proteins fall within the normal range, it may usually be assumed that there is no inflammatory response. This is especially true in patients with more than one day's fever. However, in chronic rheumatoid arthritis small nodules and synovitis may be found without any clear-cut inflammatory response of the plasma proteins. Varying patterns of the acute phase reactants are given in figure 3.

The importance of the concentrations of IgG, A, and M will be discussed by others, but it can be stated that IgG and IgA should be determined routinely and that their levels should be evaluated in conjunction with the acute phase reactants taking account of the length of the history.

The various components of complement must also be considered in the discussion of the inflammatory response. We have found that naked-eye inspection of the electrophoretic strip immediately indicates whether C3 is normal or not. Since C3 is a slow-reacting acute phase protein, values may be either normal or increased in acute illness. During subacute or chronic inflammation C3 should be increased. If not, increased complement activation should be suspected. Measurement of C3 gives little information unless levels are low, as in diseases with an antigen-antibody reaction in the circulation. C4 is another complement factor which normally increases during inflammation. Its variation is of limited clinical value and it need not be estimated routinely. It often disappears selectively in haemolytic anaemias and its measurement is therefore of value when investigating the cause of reduced haptoglobins.

Caeruloplasmin is another protein which we have measured routinely. Its estimation is of very limited diagnostic value but increased levels may suggest the administration of oestrogens or pregnancy. The combination of high α1-AT and caeruloplasmin helps to distinguish viral hepatitis from liver dysfunction induced by oestrogens.

The clinical chemist can indicate not only whether the electrophoretic strip and immunochemical results are consistent with the clinical diagnosis but can also interpret the protein changes in terms of basic pathophysiological processes.

References
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*J Clin Pathol* 1975 s1-6: 22-26
doi: 10.1136/jcp.s1-6.1.22

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