IMMUNOLOGICAL TECHNIQUES IN THE STUDY OF BIOCHEMICAL PROBLEMS

E. J. WOOD

M.A. (Oxon.)

Department of Physiology,
Royal University of Malta

Introduction

For many years now serological differentiation of bacterial strains and determination of blood groups have been standard methods in hospital laboratories. More recently a great deal of research effort has been directed towards investigating the many protein components of serum and their relation to disease. This work has been very dependent on immunological techniques. Because of their high specifity and sensitivity, such methods have become useful tools to biochemists and protein chemists. The remarkable sensitivity of some of these methods may be illustrated by the fact that the radio-immunoassay for insulin is claimed to detect amounts of insulin as small as 0.00005 micrograms (Schwick, 1968). Even without the use of radio-isotopes very small amounts of proteins can be detected: for example, by immunodiffusion, immuno-globulins in serum can be detected in 0.0025 microgram quantities.

In this laboratory immunological methods have been used to investigate problems of comparative biochemistry, to obtain estimates of the size of proteins, and to gauge the effect on protein structure produced by procedures which chemically alter the amino acids of the protein. The work in this laboratory is concerned with the study of copper-containing proteins from the blood of invertebrates (haemocyanins), and from humans (caeruloplasmin). Some of this work is described below. The principles behind the techniques are described in some detail in the hope of stimulating their use in other fields of research.

Double Diffusion in Agar Gel

If two wells a few mm. apart are cut in a uniform layer of buffered agar gel on a microscope slide, and antigen is placed in one well and antibody in the other, then in the course of 12-72 hours, the proteins diffuse outwards from the wells into the
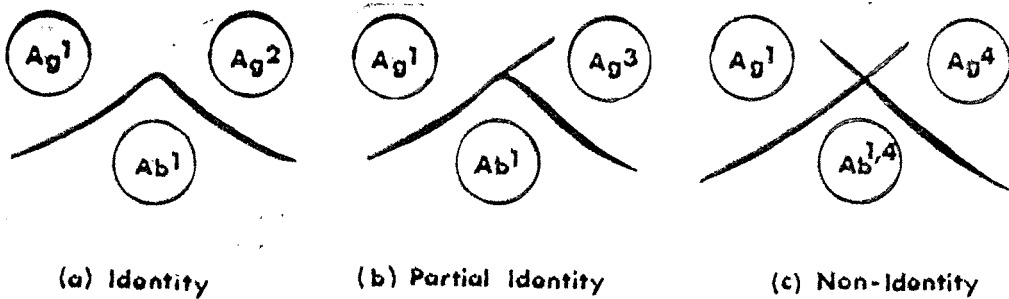
(a) Identity   (b) Partial Identity   (c) Non-Identity

FIGURE 1

Increase in Optical Density (O.D.) of a haemolysate solution obtained from baby (curve 1), mother (curve 2), father (curve 3) and a normal (control) person (curve 4) is plotted against time (minutes).

Cross-reaction in agar gel immunodiffusion. Ag^1-Ag^4 represent antigens, and Ab^1-Ab^4 the corresponding antibodies to them. (a) If the antigenic determinants of Ag^1 and Ag^2 are identical, then the outward diffusing antigens present a V-shaped advancing front to the antibody which reacts with both to form a V-shaped precipitation line. (b) If some of the antigenic determinants are common to both antigens, then a characteristic "spur" develops — the reaction of partial identity. (c) If there are no antigenic determinants common to the two antigens, then they each react independently with their antibodies, hence the precipitation arcs cross.

agar gel. When antibody meets antigen in the agar, a precipitate will form and move no more. After a day or two it is possible to see lines of precipitate in the agar, and they may be stained or photographed for a permanent record. This is a simple method of detecting antigens or antibodies in a given body fluid.

More usually it is used to detect cross-reactions between proteins. The patterns that may be obtained are shown in *Fig. 1*: the legend to this figure explains how the patterns develop.

An example of the use of this technique in our work is the comparison of the antigenic structure of haemocyanins from different species of molluscs. An antibody to purified haemocyanin from the whelk, *Murex trunculus*, was raised in rabbits (Wood *et al.,* 1968b). This antibody produced a strong precipitin line in agar against *M. trunculus* haemocyanin. A reaction of identity *(Fig. 2)* was produced against the same haemocyanin from which the copper had been removed (apo-haemo-

cyanin): thus the copper-binding site does not appear to be very important in the immunological activity of haemocyanin. Furthermore it would seem to show that removal of the copper atoms from the protein does not change the structure of the protein very much ,certainly not enough to disrupt the arrangement of the amino acids in the antigenic sites of haemocyanin.

The whelk, *Murex brandaris* closely resembles *M. trunculus*. In many cases close inspection is necessary to determine which species one is handling. It is therefore not surprising that haemocyanin from *M. brandaris* produced a reaction of partial identity to *M. trunculus* antibody. Thus the haemocyanin from the two species of whelk are closely similar but are not identical. Two other haemocyanins were investigated, both from marine molluscs with some resemblance to the *Murex* species. These two species, *Euthria* and *Cerithium* did not react at all with the antibody to *M. trunculus* haemocyanin.
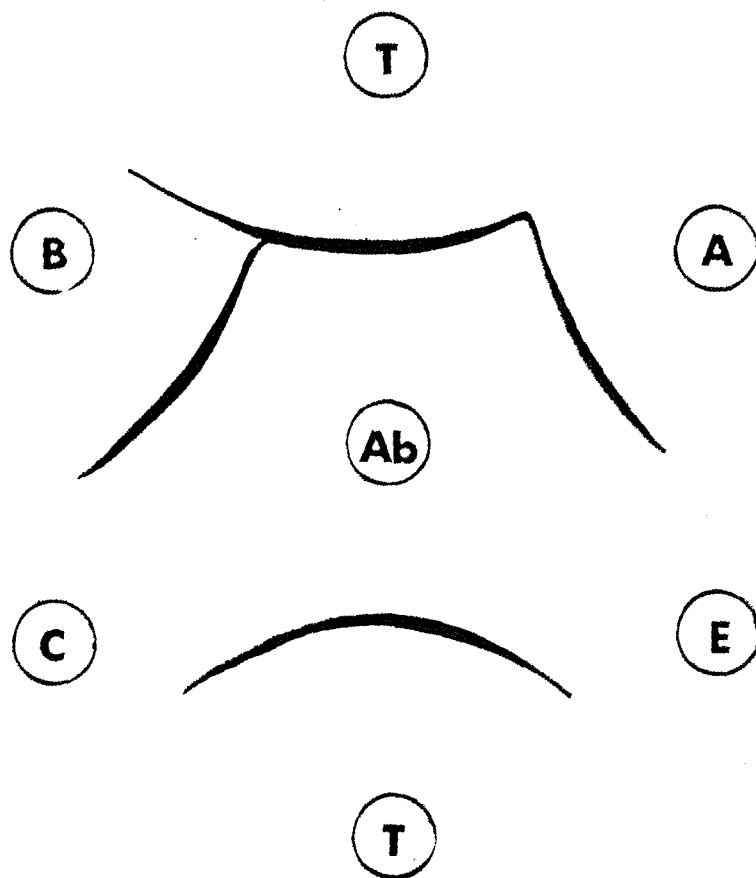
FIGURE 2
Immunodiffusion in agar gel. Centre well contains antiserum to Murex
trunculus **haemocyanin. Radial wells:**
    T: M. trunculus **haemocyanin A:** M. trunculus **apo-haemocyanin;**
B: M. brandaris **haemocyanin; C.** Cerithium vulgarum **haemocyanin;**
E: Euthria cornea **haemocyanin.**

Microimmunoelectrophoresis

Double diffusion in agar gel is thus a useful technique. However, if pure antigen, or alternatively monospecific anti-serum, is not used then several precipitation arcs may form and may be superimposed. If the antigens present in a complex mixture are first separated by electrophoresis and then are allowed to react in agar with an antiserum to the mixture several precipitation arcs arise. These can usually be identified as distinct antigen-antibody systems.

The technique of performing micro-immunoelectrophoresis is briefly as follows (see Grabar and Burtin, 1964). A 2-3 mm. layer of buffered agar is poured on a microscope slide and allowed to set. A small hole is cut and filled with antigen mixture. Wet filter paper strips are placed at either end of the agar and are dipped into buffer vessels. By means of electrodes in the buffer vessels a potential is applied across the agar layer. The antigens in the mixture separate so that after 60-90 minutes one obtains bands of antigen in the agar, their individual position, depend-
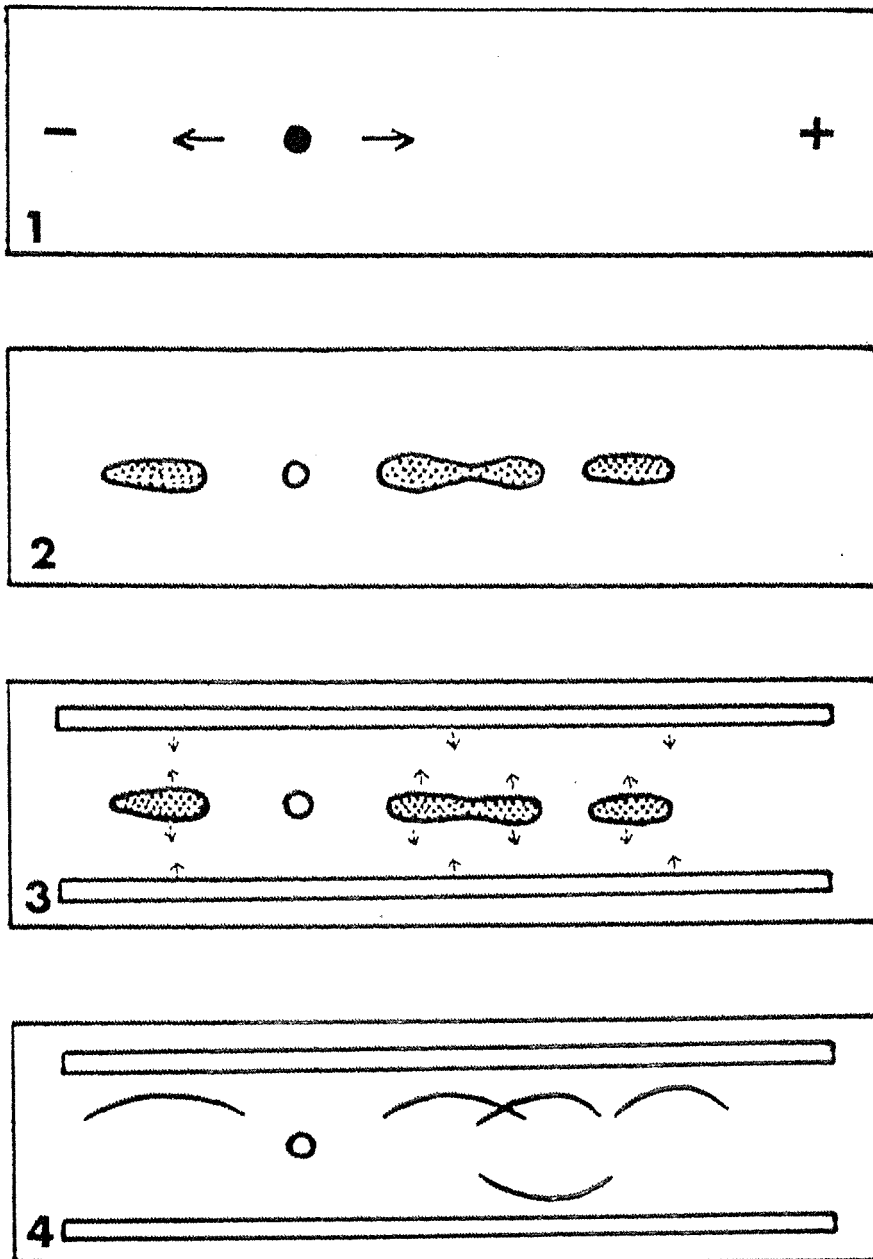
FIGURE 3
Technique of microimmunoelectrophoresis

(1) Antigen mixture submitted to electrophoresis in agar gel.

(2) Antigen mixture is thus separated into bands in the agar, the position of which depends on the electrophoretic mobility of the components. (Bands are not visible at this stage.)

(3) Troughs are cut and filled with antiserum. Antigen and antibody diffuse towards each other as shown by the arrows. The top trough contains a multivalent antiserum; the bottom trough a monospecific antiserum to one component of the antigen mixture.

(4) After 12-48 hours precipitin arcs become visible in the agar.

ing on their electrophoretic mobility. Troughs are next cut alongside these bands (see Fig. 3) and these are filled with anti-serum. After 24-96 hours antibody from the troughs has diffused towards the centre of the agar layer, while antigen from the bands has diffused outwards. Where antigen meets antibody a precipitation arc is formed. Such arcs are visualised by oblique illumination or by staining with a protein dye. The procedure is illustrated step-by-step in Fig. 3. By means of this technique up to 30 components have been demonstrated in normal human serum.

The use of monospecific sera will allow individual antigens to be picked out as shown in Fig. 3. However, monospecific sera are troublesome to make — one usually needs to prepare a single, pure antigen in sufficient quantities for injection into animals — and therefore expensive. Sometimes this can be overcome by absorption of non-specific antibodies. In many cases, however, the simple and cheap answer is to use different staining reactions, particularly enzyme stains. This technique is applicable to many serum components e.g. lipoprotein (Oil red O stain), glycoprotein (PAS), peroxidase (benzidine), catalase (H_2O_2), cholinesterase (beta-naphthyl esters), and caeruloplasmin (see below).

In this laboratory we have used the staining technique of Schen and Rabinovitz (1966) to identify caeruloplasmin lines in agar gel immunoelectrophoresis. (Caeruloplasmin is the chief copper-containing protein of the serum. About 90% of the serum copper is bound in this way. In Wilson's disease-hepatolenticular degeneration — when signs of copper toxicity appear, the serum levels of caeruloplasmin are normally very low.) The function of caeruloplasmin is uncertain, but it does have oxidase activity and it is this activity which allows the precipitin lines to be stained selectively. The agar plates are treated with ferrous salts. The caeruloplasmin oxidises these to ferric salts, which are then used in the Prussian blue reaction. Thus when a rabbit antiserum to whole human serum is run against human serum in immunoelectrophoresis, and the

plates stained by this reaction, the caeruloplasmin line appears a strong blue colour while all the other lines are white. Thus during the purification of caeruloplasmin (Stokes, 1967) from whole serum, the purity can easily be checked at the various steps by immunoelectrophoresis. In serum, and in partially purified preparations, several lines are produced, but when the final pure, crystalline protein emerges from the last step in the purification procedure, it gives a single, well defined line in the agar.

Another example of the use of immunoelectrophoresis comes from our work of trying to identify the groups in the caeruloplasmin molecule responsible for binding the copper and enzyme activity (Wood, et al., 1968a). In the course of this work the protein was subjected to photooxidation in the presence of methylene blue. This treatment resulted in loss of enzyme activity. Two explanations were possible for this. Photooxidation had either denatured the protein so that its structure was completely disorganised and hence enzyme activity lost, or it had specifically attacked residues in the active site of the enzyme causing loss of activity. Immunoelectrophoresis helped in the decision between these alternatives. Photooxidized caeruloplasmin was run against a specific rabbit anti-caeruloplasmin serum. A single precipitation arc was obtained which did not stain for enzyme activity. Furthermore the mobility of the protein was changed; in fact photooxidized protein moved faster in electrophoresis than native protein. The conclusions that could be drawn from these results are as follows. Photooxidation had caused some modification to the protein because (a) it lost its enzyme activity, and (b) its mobility in electrophoresis changed. The protein had probably not however been denatured since it still reacted with a specific antiserum to it. (It is assumed here that the antigenic site(s) within the protein molecule, like the enzyme site, are dependent on the persistence of the normal three-dimensional structure of the polypeptide chains of the protein. What one usually understands by denaturation, for example, that produced

TABLE

Changes in serum proteins in certain pathological conditions

Disease	Change observed
Wilson's disease	Caeruloplasmin line weak or absent.
Agammaglobulinaemia	γ-globulins low or absent.
Myelomas	γ-globulins increased, esp. Bence-Jones protein = unique protein in β-γ region.
Waldenstrom's disease	atypical β2M-macroglobulin.
Lupus erythematosus	albumin decreased, α^2-globulin increased (esp. haptoglobulin), β1A/C-globulin decreased.
Rheumatic disease	C-reactive protein (acute phase protein β1-globulin) appears.

by heating the protein, is a complete disorganisation of the three-dimensional structure, when any specific enzymic or antigenic sites will be destroyed.) The conclusion therefore was that the effect of photooxidation was on certain amino acid residues in the active site. By means of amino acid analysis it was possible to identify which amino acids had in fact been changed by photooxidation.

Comments

It will be seen from these examples that immunological techniques have considerable potential in biochemical investigations. The methods are simple and rapid to perform, and have great sensitivity and specificity. Furthermore they may be made quantitative. Reference has already been made to the immunossay for insulin. In fact agar gel diffusion may be used to measure the amount of almost any antigen, for example a serum protein and its variation in disease, by means of the Heremans and Mancini (Mancini et al., 1964, 1965) technique of radial immunodiffusion. The beauty of the method lies in the fact that the antigen does not have to be purified: the specificity of the antiserum is such that other proteins that may be present, even in large quantities, do not interfere. In this method a layer of agar is prepared which contains the specific antiserum uniformly distributed through it. Wells are cut and filled with antigen. As the antigen diffuses outwards it meets antibody in the agar, so that eventually a ring of precipitate is formed. The diameter of this ring is directly related to the amount of antigen in the well.

Even in the standard technique of immunoelectrophoresis, qualitative and quantitative differences in the pattern of precipitin arcs are recognised in a very large number of diseases. Some of these are listed in the Table.

Acknowledgements

References

GRABAR, P., and BURTIN, P., (1946). "Immunoelectrophoretic Analysis". (Elsevier Publishing Co.).

MANCINI, G., CARBONARA, A.O., and HEREMANS, J.F., (1965). Immunochem. 2, 235.

MANCINI, G., VAERMAN, J.P., CARBONARA, A.O., and HEREMANS, J.F., (1964). Protides. Biol. Fluids: Proc. 11th Colloq. (Bruges, 1963) p. 370.

SCHEN, R.J., and RABINOVITZ, M., (1966). Cl'n. Chim. Acta. 13, 537.

SCHWICK, H.G., (1968). 6th Int. Congr. Clin. Chem. (Munich, 1966) Vol. 1, p. 93.

STOKES, R.P., (1967). Clin. Chim. Acta, 15, 517.

WOOD, E.J., SALISBURY, C.M., and BANNISTER, W.H., (1968a). Experientia, in the press.

WOOD, E.J., SALISBURY, C.M., FORMOSA, N., and BANNISTER, W.H., (1968b). Comp. Biochem. Physiol 26, 345.