Present-day practice

Principles of protein estimation by radial immunodiffusion

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In descriptions of the quantitative estimation of proteins by radial immunodiffusion some authors read the results while the ring is still growing, others wait until equilibrium has been reached. While assembling the material for a review (Grant and Butt, 1970), I was struck by the absence of clear statements on the principles involved in these two different approaches and the consequent possibility of misunderstanding and error. Furthermore, principles are not considered in the otherwise excellent A.C.P. Broadsheet by Professor Hobbs (1970).

In 1964 Heremans and his colleagues (Mancini, Carbonara, and Heremans, 1965) showed that the tedious, wasteful, and imprecise endpoint methods current at the time were unnecessary: the concentration of a serum protein could be estimated immunochemically from a single dilution if this were placed in a cup cut in a plate of antiserum-containing agar and the final size of the precipitin ring formed were compared with those formed by suitable standards.

The precipitin ring after it first becomes visible keeps on being redissolved by excess antigen and reforming further out. Eventually, provided sufficient antiserum has been mixed with the agar, it will come to a halt. The serum protein in the sample has now combined with an equivalent amount of antibody, namely, that which was in the agar within the ring. Its amount is therefore proportional to this area and so to the square of the diameter of the ring. This and the uniform conditions made possible by comparing unknown and standards on the same plate are the basic principles of the Mancini method. Note that the rate of diffusion of the serum protein only affects the time required to reach the stationary state, not the results, and that the ring will remain unchanged indefinitely provided that the kind of antiserum used is incapable of redissolving the precipitate, eg, rabbit or goat, not horse.

In 1965, Fahey and McKelvey published a technically similar method but used higher antigen/antibody ratios and read the diameters of the rings

before they became stationary. At this stage variations in timing, in temperature—since it affects the rate of diffusion—and in any heterogeneity of the serum protein molecules will all affect the size of the ring. The principle of the method, like that of the AutoAnalyzer, is that the reaction need not be allowed to go to completion provided that all the samples are given rigidly identical treatment, in this case by putting up standards and unknowns on the same plate.

The range of concentrations which can be estimated on a single plate is similar whichever approach is used: it is dependent, as in all gel precipitin techniques, on a balance between the amounts of antigen and antiserum used and on having sufficient quantities of them to produce a visible line.

The Fahey method, or that in which rings are measured indiscriminately, whether moving or stationary, allows neat serum to be tested without previous dilution and sometimes gives quicker results. It is the method of the commercial Hyland plates. Its disadvantages, however, are considerable.

First, it is less precise, both because the results depend on the rate of diffusion so making them very susceptible to variable conditions, and because there is no constant straight line relationship between the concentration and the ring size. As a precipitin ring forms, its diameter is directly proportional to the logarithm of the concentration; at equilibrium it is proportional to the square root of the concentration; in between there is no constant relationship. The results are bound to fall on a curve, so it is easiest simply to plot the diameter against the concentration. Numerous standards are essential: extrapolation, and even more comparison of the results of one plate with standards on another, are sure to lead to errors.

Secondly, if the results are not read early enough, the precipitin rings may become hazy and unreadable. This is because neat serum giving a high antigen/antibody ratio is used to produce a measurable ring rapidly. If not read in time there may be insufficient antiserum in the agar to react with all the serum protein antigen.

Thirdly, in order that neat serum may be used, a wasteful amount of antiserum is often employed, whereas the Mancini method is most rapid and precise as well as most economical when the smallest amount of antiserum consistent with a visible line is used.

To summarize, if it is decided to use the Fahey or Hobbs techniques, the limitations of the method should be taken into account. I believe the original Mancini approach to be preferable: in an emergency the tests can always be read in a few hours and the final results read later at equilibrium.

Suitable plates can be made locally or bought from Messrs Hoechst.

References

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