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Differing Methodology and Equations Used in Quantitating Immunoglobulins by Radial Immunodiffusion—A Comparative Evaluation of Reported and Commercial Techniques[1]

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Differing methods of plotting have been used to describe the results of radial immunodiffusion (RID). Two, logarithm of antigen concentration (log *c*) vs. precipitin-ring diameter (*d*) (Fahey) and log *c* vs. area (*A* or d^2) (Mancini), are linear while rings are enlarging. Another, *c* vs. *A* (or *c* vs. d^2), becomes linear when enlargement ceases at equivalence (Mancini). Because of methodological inaccuracy, I could not experimentally determine whether $\log c = d$ or $\log c = d^2$ is appropriate; either one or both semilogarithmic graphs may be linear as precipitin circles enlarge. I could confirm the $c = d^2$ linearity at equivalence. Because smaller circles cease growth first, intermediate readings produce plots that are partially curved and partially straight. Commercial RID plates produce linear and curved plots of log *c* vs. *d* and of *c* vs. d^2. They often create too small a diameter range for great accuracy. I conclude that semilogarithmic plots are of questionable value. For greatest accuracy plates should be designed for measurement at equivalence. At this time, a straight-line graph of $c = d^2$ will include a maximal diameter range.

The scientific literature contains several discrepant equations that reportedly describe the radial immunodiffusion (RID) of antigen into antibody-containing agar (*1*). Because some of these lead to confusing or erroneous conclusions, I decided to reexamine the situation by experimental techniques and to survey the product literature of the commercial RID plates on the market.

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[1] We dedicate this work to Drs. Örjan Ouchterlony and Joseph F. Heremans, who with their collaborators transformed radial immunodiffusion from a laboratory curiosity into a tool for the hand of every clinician and scientist.

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In 1905, Bechhold, working in Paul Ehrlich's laboratory, overlaid goat serum onto the top of a solidified mixture of gelatin and an antiserum produced in a rabbit. He observed the formation of two precipitation zones, which he considered analogous to the concentric rings of precipitate formed when he placed a solution of inorganic reagents into the center of a plate of gelatin mixed with other reagents (*2*). (Although he attributed the dual antigen-antibody reactions to the "Liesegang phenomenon" of inorganic reactions, it is more likely that the two zones resulted from the precipitation of two different antigens in the goat serum.) In 1932, Petrie described circular precipitates surrounding toxigenic bacteria growing in a dish of agar containing antitoxin (*3*); others made similar observations.

Ouchterlony (*4*) reviewed the earlier literature in 1949 and published a graph of relationships he had observed in the radial diffusion of diptheria toxin into antitoxin-agar. He concluded that this technique could form the basis of a quantitative test for antigen and antibody, because the production and size of the precipitin halo depended upon the concentration and potency of the reagents. Others (*5, 6*) extended the quantitative methodology of RID during the following decade.

In 1963-1965, Mancini et al. in Heremans' laboratory (*7-9*) and later Fahey and McKelvey (*10*) provided the impetus toward the popularization of RID by observing certain relationships between the concentration of the antigen and the size of the precipitin ring. It is not widely recognized that the methods and results of these two groups are not in concordance. Most European workers use the technique of Mancini et al.; those in the U.S. use that of Fahey, perhaps reflecting the geographic origin of the methods.

Before RID was introduced as a quantitative tool, Oudin had described the usual relations occurring in the vertical migration of a precipitation zone into antibody-agar in tubes (*11, 12*) observing a form of the equation $\log c = h/\sqrt{t}$ (slope and intercept constants are omitted throughout; mathematical relationships should be regarded as proportionalities rather than as true equalities), where c is the initial antigen concentration, h is the height (penetration distance of the precipitation zone), and t is the time elapsed. Becker et al. (*13*) found that another relation, $\log c = h^2/t$, occurs when antigen is greatly in excess of antibody. If tubes containing different concentrations of antigen are read after the same elapsed time, these relations simplify to $\log c = h$ and $\log c = h^2$, respectively. With these simplified relations, experimental data can be plotted as straight lines on semilogarithmic graph paper, with either h or h^2 as one axis. They exist only so long as the precipitation zone continues to migrate. The value of h also depends on the temperature and the diffusion coefficient of the antigen. It must be recognized that these straight lines reflect the diffusion rates of the antigen and consequently the migration rates of the precipitation zone under different conditions. The relation $\log c = h$ holds true while antigen and antibody diffuse horizontally toward each other (double diffusion) from wells in an agar plate while antigen is in excess and the precipitation zone (observed as a band) is traveling toward the antibody well (*14*).

Mancini et al. (*8*) considered that Oudin's $\log c = h$ would transform in RID into $\log c$ = area of the precipitin ring; this is equivalent to $\log c = d^2$, where d is the diameter, since area $= \pi d^2/4$. Their experiments demonstrated that such a relationship indeed briefly exists after antigen is applied.

On the other hand, Fahey and McKelvey (*10*) observed that the 24-h refrigerated precipitates of several antigens followed a linear plot of $\log c = d$. Although not so mentioned by Fahey, later writers (*15, 16*) postulated that this would represent the transformation of Oudin's $\log c = h$, in contrast to Mancini's concept. If this is correct, Mancini's $\log c$ = area (or $\log c = d^2$) would actually represent a transformation of Becker's relation ($\log c = h^2$) rather than Oudin's $\log c = h$.

In addition to attempting to formulate equations describing enlargement of the circles, Mancini found that precipitin rings in RID eventually stop expanding when the antigen is depleted and antigen–antibody equivalence occurs. The smaller circles are those that contained less antigen and thus were the first to stop enlarging. Mancini described an equivalence relation, c = area (which converts to $c = d^2$) and plotted a straight-line graph of this proportionality. Measurements at equivalence were found to be independent of temperature, diffusion rate, and other complicating variables that influence the size of circles that are still enlarging.

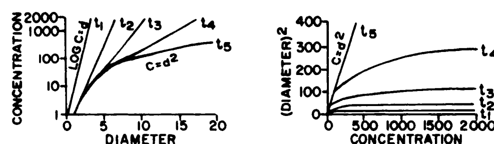

Fig. 1. Theoretical graphs relating antigen concentration (*c*) to precipitin ring diameter (*d*) in RID

It is assumed that the relationship $\log c = d$ occurs during migration of the precipitin zone (as per Fahey), that $c = d^2$ when migration has ceased at equivalence (as per Mancini), and that the smallest circles cease enlarging first. *Left*, semilogarithmic plots of c vs. d. *Right*, plots of c vs. d^2 on linear scales. t_1–t_5 are increasing time intervals from antigen application to measurement of precipitin ring

Other relations such as $c = d$ (*17–20*) and $\log c = \log d$ (*21, 22*) also appear in the literature; the former has never been well documented and, as Kalff has noted (*16*), the relation $c = d^2$ is nearly linear when one plots c vs. d on log-log paper, explaining the latter.

If $\log c = d$ (Fahey) expresses the relationship during expansion of the precipitin ring as the precipitation zone migrates and $c = d^2$ (Mancini) occurs at an end point reached first by precipitin circles containing the smallest amount of antigen, observed plots will be consistent with the theoretical graphs of antigen concentration vs. diameter shown in Figure 1, where t_1–t_5 are increasing time intervals from antigen application. Linearity ($\log c = d$) occurs early on the semilogarithmic plot (*left*) and is progressively lost by circles produced by increasingly larger antigen concentrations as time elapses; the curve is completely nonlinear when all circles reach their end points. Conversely, the (diameter)^2 plot (*right*) becomes progressively linear as time elapses until all circles fall on the straight line produced by $c = d^2$ at the termination of enlargement.

The following experiment was designed to re-determine the relationships obtained from RID. The results tend to confirm the theoretical plots described above. I also describe the plots reported in instructions accompanying commercial products and compare these with the observed and theoretical curves.

Materials and Methods

A monospecific goat antiserum to rabbit IgG was suspended at 51 °C in 0.15 g of "Special Agar—Noble" (Difco Laboratories, Detroit, Mich. 48201) per liter dissolved in a mixture of, per liter, 0.16 mol H_3BO_3, 0.13 mol of NaCl, and 35 mmol of NaOH buffer (pH 8.0), with 0.2 g of Na_3N added as a preservative. The antibody-agar was poured into rectangular 28 × 17 cm plates to a depth of 4 mm, after a 4-mm layer of agar dissolved in water (30 mg/liter) had dried into a thin film coating the plates. Forty 3.4-mm wells, with centers 20 mm apart, were punched into the antibody-agar after it had solidified. Ten dilutions of a reference rabbit serum in buffer, ranging from 0.05 to 0.95 ml of reference serum per milliliter of diluted serum, were placed

into various wells; thus, antigen in the most concentrated serum was 19-fold as concentrated as in the most dilute.

Antigen concentrations were arranged so that adjacent reactions did not interfere with each other. With capillary tubing, antigen was introduced into each well until the surface was flat and level with the top of the agar, thus minimizing difficulties that might be caused by local irregularities in the depth of the agar. (This technique produced more consistent results with these plates than was the case when a constant volume was used per well.) Each antigen concentration was applied in quadruplicate to each plate.

The plates were stored in humidity chambers at room temperature (about 27 °C) and the circles were repeatedly measured after 2 h to 10 days, by which time all circles had ceased to enlarge. After preliminary tests, the antiserum concentration was adjusted to produce precipitin rings having a final maximum diameter ranging from 5 to 17 mm. Two measurements across the tops of the precipitin cylinders (rings) were made at right angles and averaged. After testing various rulers and magnifying comparators, I found that an angular template obtained from Behring Diagnostics gave the fastest and most accurate readings of diameters and their squares. (The vendor claims this to measure the ring diameter; it actually measures a line through the circle that is shorter than this. However, the resulting error is small and was neglected in recording results.) The quadruplicate experiment was repeated six times.

Product literature on commercial RID plates was kindly supplied by Behring Diagnostics, Somerville, N.J. 08876; Hyland Division, Travenol Laboratories, Costa Mesa, Calif. 92926; Kallestad Laboratories, Chaska, Minn. 55318; Lederle Diagnostics Division of American Cyanamid Co., Pearl River, N.Y. 10965; Meloy Laboratories, Springfield, Va. 22151; Miles Laboratories, Kankakee, Ill. 60901; Oxford Laboratories, Foster City, Calif. 94404; Pfizer Diagnostics, New York, N.Y. 10017; Wellcome Reagents, Beckenham, Kent, England; and Dade Division of American Hospital Supply Corp., Miami, Fla. 33152.

Experimental Results

Precipitin rings were difficult to measure reproducibly at early times, but became progressively sharper until such time as they ceased to enlarge (equivalence). Equivalence was reached within 2 h for the lowest (0.05 ml/ml), 140 h for the highest (0.95 ml/ml) antigen concentrations. Maximum variation of the quadruplicates was about 10–20% up to 28 h for those circles that were enlarging, diminishing with time to 5–10% at equivalence. Results of all six experiments were similar; one is illustrated in Figures 2–4. In Figure 2, the concentration is plotted as a logarithmic ordinate and the mean diameter as a linear abscissa, according to Fahey. The plots resemble the theoretical curves in Figure 1 (*left*); at first the plot is linear, but it progressively becomes more curved for increasing concentrations as time elapses. The slope of the linear portion decreases with time; thus, with increasing time, differing concentrations produce more markedly different diameters as the circles enlarge. By 140 h, even the largest circles are no longer enlarging and the curve is completely nonlinear. In these graphs, linear portions are interpolated; in some instances the lines do not pass through all points, particularly in the earlier readings.

The same data are plotted semilogarithmically in Figure 3; here, the squares of the diameters are plotted on a linear abscissa, in accordance with

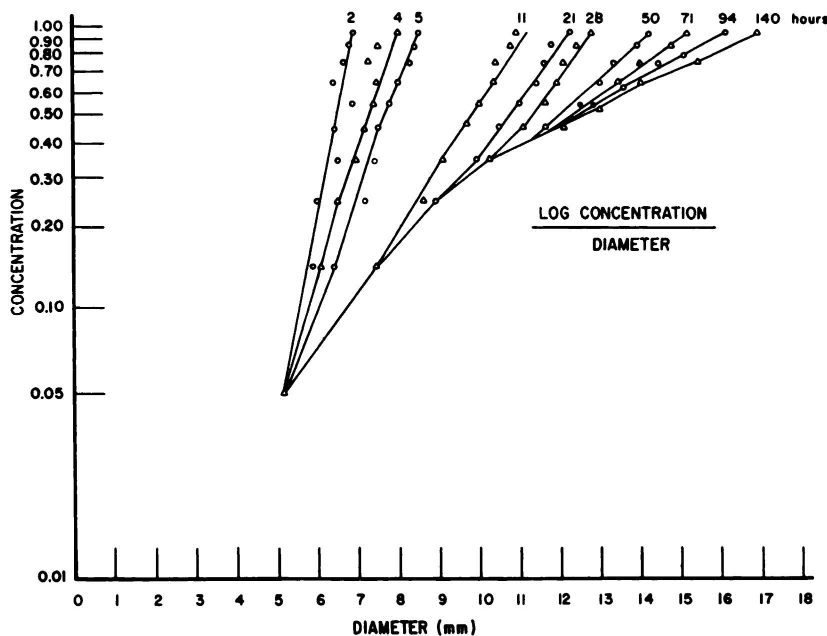

Fig. 2. Experimental results of RID plotted as semilogarithmic graphs of antigen concentration vs. precipitin ring diameter as per Fahey and Figure 1 (left)

Measurements made at increasing time intervals from antigen application until all circles have ceased growth

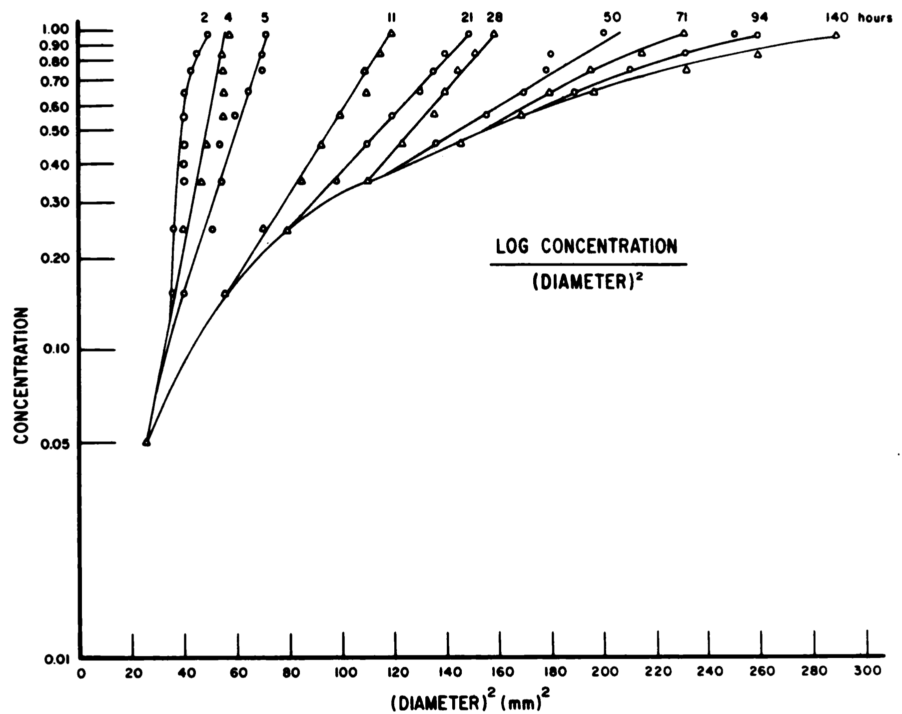

Fig. 3. Experimental results plotted as semilogarithmic graphs of antigen concentration vs. square of precipitin ring diameter corresponding to semilogarithmic plots of antigen concentration vs. precipitin ring area (Mancini)

Mancini's concept of precipitin migration. As in Figure 2, the "best" curve is a straight line while circles are enlarging. This is more readily apparent at early times; at 50–94 h, no reasonable interpolation gives a straight line. The early readings exhibit a relatively narrow range of diameters; the probability of a coincidental deviation from linearity decreases with time as the range of diameters increases relative to the concentration range.

None of the plots in Figures 3 or 4 are linear for a wide range of diameters. As time proceeds, the curved lower portion of the plots occupies an increasingly larger part of the range; this portion contains those circles that have ceased growth.

In Figure 4, the squares of diameters are plotted as a linear ordinate and the concentrations as a linear abscissa as in Figure 1 (*right*). The results resemble the theoretical graphs and those of Mancini; here, linearity appears in progressively larger portions of the curve, corresponding to cessation of growth. The plot is linear over the entire range at equivalence; nearly all points fall on the end-point line, attesting to the accuracy of the procedure.

Concentrations were also plotted vs. diameters on linear scales. No straight lines ($c = d$) appeared over an extended range of diameters. In contrast, plots of c vs. either d or d^2 on log-log paper were linear at all times, primarily because the range is exponentially compressed in this procedure, which reduces sensitivity.

Review of Commercial Literature

For commercially supplied RID plates (see above), the suppliers recommend a variety of plotting techniques (Table 1). The instructions provided with each do not always state whether measurements are to be made during or after enlargement; only Behring, Oxford, and Wellcome consider both possibilities. Behring recommends that measurements be made at equivalence, Wellcome suggests an earlier reading and Oxford discusses both (see below).

All except Behring recommend plotting concentrations logarithmically on semilog paper; Kallestad, Lederle, and Pfizer require that a straight line of best fit be drawn with all plates, and Hyland requires such a line in some cases. Behring requires a straight c vs. d^2 line when its plate is used at equivalence. Oxford requires a straight semilogarithmic line for measurements after a short specified time, but suggests that greater accuracy can be obtained by constructing a straight $c = d^2$ line after precipitin rings stop enlarging. This is the only vendor in this

Table 1. Commercial Radial Immunodiffusion Plates (1972–1973)

Vendor	Measure at equivalence	Vendor's suggested graph: Scales	Vendor's suggested graph: Draw best straight line
Behring	yes	d^2/c	yes
	no	d^2/c	no
Oxford	yes	d^2/c	yes
	no	log c/d	yes
Wellcome	yes	log c/d	no
	no	log c/d	no
Kallestad Lederle Pfizer	no	log c/d	yes
Hyland Meloy	no	log c/d	yes/no
Miles Dade	no	log c/d	no

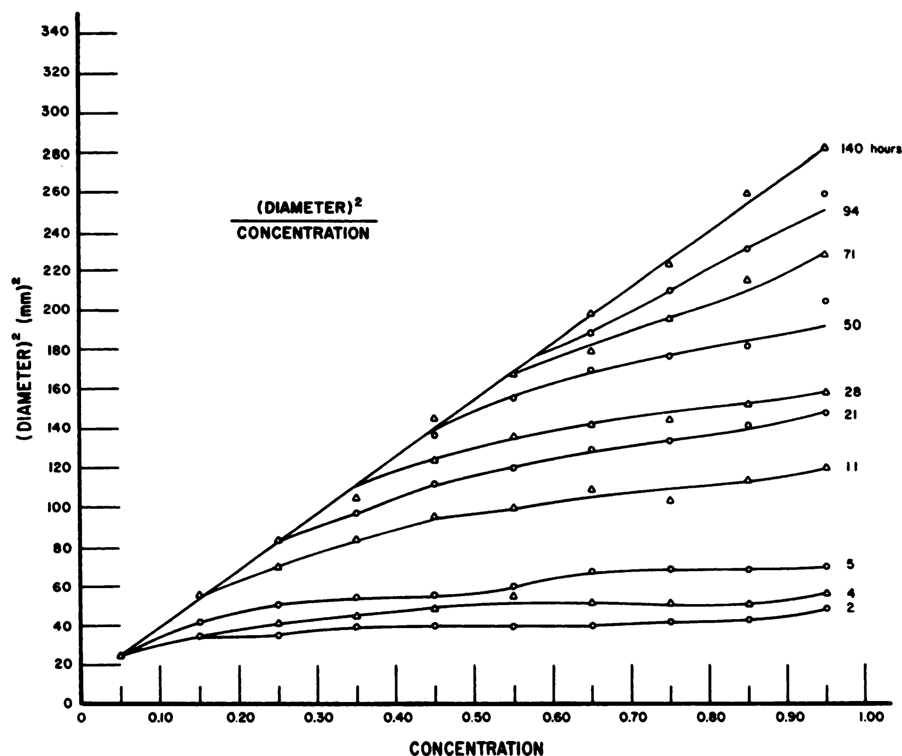

Fig. 4. Experimental results plotted as antigen concentration vs. square of precipitin ring diameter on linear scales as per Figure 1 (right) and corresponding to plots of antigen concentration vs. precipitin ring area (Mancini)

survey that makes use of both the Mancini and Fahey techniques.

In the supplier's literature, there are examples of each theoretical curve; Kallestad, Lederle, Oxford, Pfizer, Hyland (high-level IgG), and Meloy (low-level IgA) show the complete semilogarithmic straight line of t_1 and t_2 in Figure 1 (*left*); Hyland (low-level IgG) and Meloy (IgG, high-level IgA) show semilogarithmic lines that are curved at the bottom and straight above (t_3 and t_4). Meloy also illustrates the complete semilogarithmic curve of t_5 (IgM, IgD), and Behring shows the linear relation for square of diameter vs. concentration of t_5 (Figure 1, *right*) that occurs at equivalence as well as a nonlinear curve for earlier measurements (t_1–t_4). None advises that squares of diameters or areas be plotted on semilogarithmic paper, nor do any show curves that are not included in the theoretical plots.

All vendors providing illustrations show only a narrow range of diameters, typically 5–8 mm, in contrast to the wide range (5–17 mm) demonstrated in the experiments reported here. Manufacturers suggest plotting 3–5 points to create a curve; three points are invariably suggested to create a straight line of best fit. Most (but not all) caution against extending lines or curves past the terminal points. All specify the temperature and diffusion time.

Discussion

The investigations I describe demonstrate that several linear plots may occur in RID. It does not seem possible to decide from these experiments whether $\log c = d$ or $\log c = d^2$ better represents the migrating precipitation zone. Either kind of plot gives straight lines for 11 h for all concentrations and longer for the higher ones. The failure to establish a definite migration relationship stems from three factors: (*a*) Experimental inaccuracy during the early stages of diffusion, owing to the narrow range of diameters being measured and the diffuseness of the precipitin rings. The differences between d and d^2 are small in a narrow range. (*b*) The range of linearity on semilogarithmic plots is restricted after prolonged diffusion because the smaller circles, which lie on the nonlogarithmic $c = d^2$ line, have stopped enlarging. (*c*) It may be that $\log c = d^2$ actually expresses the relationship for the higher initial concentrations, where antigen is in extreme local excess at an early stage. The relationship $\log c = d$ may apply at these concentrations after two-dimensional diffusion has dispersed the antigen and reaction with antibody has reduced it. This change in relation will occur if the plate is large enough to contain sufficient antibody to prevent its depletion.

When enzymes diffuse radially into substrate-agar, the diameters of the circles of digestion can be related to the initial enzyme concentration by a straight-line plot of $\log c = d$, as Mestecky et al. (*23*) demonstrated for amylase in starch-agar and Kaplan and Austen (*24*) showed for plasmin in fibrin-agar. Because enzyme, unlike antigen, is not depleted by its reaction, the circles become very large and continue to grow until all the substrate is digested. In this system, therefore, a wide range of diameters can be measured while all are increasing, in contrast to the situation in RID, where growth ceases at equivalence, which is attained after various periods of time, depending on the amount of antigen present. For en-

zymes, this wide range makes it possible to plot accurate straight lines relating diameter to enzyme concentration on semilog paper, but this is not so for RID. The kinetics of migration of enzyme and of precipitation zone may not be completely analogous. If they are, the Fahey line (log $c = d$) would be the proper expression of migration in RID.

These experiments confirm Mancini's observation that smaller circles reach equivalence first in RID. Indeed, it can be reached as soon as they become visible if the antigen concentration is low enough relative to the concentration of antibody.

Mancini's relation of c = area ($c = d^2$) is clearly appropriate at equivalence. The straight line that appears on the graph of c vs. d^2 passes through nearly all points representing those circles that have attained their end points and ceased growth. In contrast, semilogarthmic plots curve throughout their entire courses at this time.

Accuracy improves with time, primarily owing to the greater differences in diameters between different antigen concentrations at later times. The existence of a true linearity ($c = d^2$) extending over a wide range at equivalence increases accuracy when one is constructing a standard curve. In addition, diameters at equivalence are independent of temperature and local variations in diffusion rates; they can be read at any time after reaching their end points. If the plate contains sufficient antibody, circles are most distinct at this stage. All of these factors combine to create an accuracy resulting in an intraplate variation of less than 10% at equivalence. The residual experimental error stems largely from pipetting inaccuracy in preparing dilutions and in filling the wells.

The literature contains several sophisticated theoretical analyses of immunodiffusion (*13, 25–31*), but none seems adequate to account for all of the relationships that are actually found in RID. There is a particular need for a rigorous mathematical model capable of deciding whether the Oudin relation converts into log $c = d$ or log $c = d^2$. The mathematical conversion of an immunodiffusion equation from one to two dimensions generates a Bessel function (*25*).

Last year, we documented some of the confusion extant in the scientific literature of RID (*1*). As an additional example, Weiner and Zak (*32*) recently illustrated the straight-line semilogarithmic plot of log $c = d$ of Fahey in a book on standard clinical methods, but rather than crediting Fahey, they cited only Mancini et al. (*8*), who had measured areas, not diameters. Further, Weiner and Zak instructed the reader to construct a straight line of best fit through three points—without specifying time, temperature, diffusion coefficient, equivalence, antigen, etc. As shown above, this would cause an inaccurate representation if any of the circles had ceased to enlarge and would lead to considerable improvisation if all had. The illustrated points (*32*) have the narrow range of 9–12 mm; the authors extended the line past the terminal points in both directions. Extending such a plot could only accentuate the error resulting from an artificial conversion of a curve into a straight line. This "standard method" appeared some five years after the publication of papers presented at an international symposium on the standardization of immunological methodology. At that 1965 meeting, Soothill and Rowe (*33*) had reported that linearity could not be obtained on the Fahey graph 24 h after the application of several different antigens, including the same ones that Fahey had used. Their curve is essentially the same as those at t_3 and t_4 in Figure 1 (*left*). Additionally, two groups had independently observed the actual transformation of the semilogarithmic straight line into a curve in 1965 and 1966 (*34, 35*) and Kalff had proposed a theoretical explanation for this in a 1970 review (*16*).

A 1972 procedural guide for the measurement of immunoglobulins produced under the auspices of the U.S. Public Health Service illustrates a semilogarithmic curve for a small range of diameters (*36*). The plot curves in the expected direction but, inexplicably, is more linear at the bottom than at the top. This is presumably due to methodological inaccuracy inherent in procedures not based on equivalence determinations. The plot differs from that recommended in a work sponsored by the American Society of Clinical Pathologists in 1972 (*37*). The ASCP guide, like that of the AACC (*32*), directs that the best semilogarithmic straight line be constructed through three points, and extends it past the determined range.

A review of commercial product information shows that several plotting techniques are in use. Each of these is consistent with one or another of the curves observed in the present experiment; the differences among them are a reflection of whether some or all of the circles are still enlarging at the time of measurement. All commercial RID plates are designed for rapid reporting of results. None (except possibly Oxford's) allow sufficient time or space for a prolonged diffusion to create a wide range of diameters. Even the "Tri-Partigen" equivalence plates of Behring produce small precipitin rings; the product literature illustrates a plot derived from measurements of circles whose end point diameters ranged only from 5.0 to 7.9 mm. This inevitably must reduce the accuracy of the test, as does the frequent provision of too few standards (three) to plot a reliable curve.

Meloy produces plates to be used in one of two ways. Either a single standard serum is diluted two or more times and the results are plotted as a curve on semilog paper, or a straight semilogarithmic Fahey line is constructed from results obtained by using three standards under strict control of time and temperature. The latter procedure allows for a statistical evaluation of the calibration line at three points and for a quality-control program that discards plates that fail to produce a line falling within the confidence limits that the vendor provides. However, such

critical time and temperature control may be difficult to attain in the day-to-day practice of a busy laboratory. In contrast, users of the Mancini technique have no such limitation as long as equivalence is achieved; they save plates and find quality control simpler. Further, Meloy's second method is probably less accurate than the first, because none of the rings are measured at equivalence and the range of diameters is small (e.g., 6.9–10.0 mm). Additionally, the vendors' illustrated points do not necessarily describe a log $c = d$ regression line rather than another. (As might be expected from plates essentially copied from Hyland's popular model, its tests are not very accurate; we can draw straight lines that fit both $c = d^2$ and log $c = d^2$ well within the 95% confidence limits of all three points on the log $c = d$ line described for IgG in Meloy's 1973 catalog and a nonlinear curve like that of t_3 in Figure 1 passes exactly through the three points whereas the log $c = d$ line does not.)

Several authors have already documented the disadvantages inherent in measuring small circles and narrow ranges. Mancini et al. (*8*) showed that increasing the slope of the area/concentration line at equivalence also increased the sensitivity. This was achieved by diluting the antiserum in the gel while retaining the same antigen concentrations. Kalff (*16*) additionally found that the accuracy could be increased at equivalence by measuring only the larger diameters on the calibration line. To achieve a benefit from both of these procedures, it is necessary to increase the areas of the circles at equivalence. This requires that the diffusion time be prolonged before reaching the end point. It is worth noting that immunoglobulin values are often obtained for patients with chronic diseases, whose treatment is little improved by undue haste in reporting results.

The inadequacies of the Fahey method have long been known to some workers. Indeed, a 1966 publication (*38*), cited in the Hyland IgA literature for another purpose, actually states that this vendor's products were useless except for the screening of gross immunoglobulin abnormalities. The plates' dependence on early measurements was noted to decrease the sensitivity and precision to levels too low for adequate use in research. Other writers (*16, 39*) later provided excellent discussions on the advantages of the Mancini equivalence technique relative to the Fahey method.

Hosty et al. (*40*) have recently reported comparative tests of RID plates for immunoglobulins from five commercial vendors. Although none was found to be completely satisfactory, they stated that Behring plates were the most reliable of those tested. It is noteworthy that this is the only supplier in their survey that suggests measurement after all circles have reached their end points. It is also the only one that uses a straight-line plot ($c = d^2$) whose reality we can affirm without doubt.

I caution the reader against attempting to obtain equivalence on a commercial plate designed for earlier readings. As Grant (*39*) has noted, such a plate is designed to be efficient in antigen excess, and may contain insufficient antibody to react with all of the antigen. Circles may become diffuse and unreadable if allowed to enlarge past their intended sizes. Additionally, atypical dose–response relations such as $c = d$ may appear at the end points (*41*).

As the Fahey method is essentially a modification of Oudin's method in tubes (*12*), one might expect it to be as accurate when used under the proper conditions. Because the migration rate of the precipitation zone relates inversely to the antiserum concentration, the Fahey method, like the Oudin technique, functions best with dilute antiserum. This allows for a rapid enlargement of the circles to a measurable size and delays equivalence; it also conserves antiserum. In addition, Oudin has noted that large excesses of antigen are advantageous during migration in tubes as precipitation zones are shorter and sharper than when the proportions of antigen and antibody are closer to equivalence. However, we have never observed this in RID; diluting the antiserum makes the precipitate less distinct and increasing the antigen seems only to prolong the time before circles become clear. Any enhancement of the rings appears only as the circles approach equivalence; additionally, the larger circles seem never to become as distinct as the smaller ones, which start closer to equivalence—probably because of the two-dimensional dispersion of the antigen. As this dispersion also hastens equivalence in contrast to the Oudin technique, the semilogarithmic straight line is far more transient in plates than in tubes. Thus, the proper application of the Fahey method requires all of the attention to details of time and temperature as the Oudin technique but cannot approach it in accuracy.

The experiments reported here deal only with IgG. While Mancini et al. (*8*) and Vaerman et al. (*42*) reported that antigens of widely different sizes produced the same relations, we must inject a word of caution regarding large molecules with small diffusion coefficients. James et al. (*17*) found that α_2-macroglobulin achieved a $c = d$ relation at equivalence, and Schmid (*43*) reported that the $c =$ area relation appeared for fibrinogen while the precipitin rings were still enlarging. Although both of these authors reported their results to be statistically significant, neither supplied enough data for evaluation by others. In contrast to the results of James et al., I have found that the relationship $c = d^2$ exists for α_2-macroglobulin at equivalence in cellulose acetate (*44*). Nevertheless, I think it unwise to completely discount their findings; aberrant plots might result if the supporting medium significantly trapped or impeded these large molecules. Those molecules capable of binding or reacting with the medium might also show different relations.

I conclude that for IgG and most other proteins, the longer one waits before making measurements before equivalence, the larger will be the range of

precipitin ring diameters and the more reliable will be the results of RID. Accuracy is maximal at equivalence, when circles have ceased growth, and depends on the attainment of a wide range of diameters relative to the concentration range. Under these conditions, one can draw a straight-line plot relating antigen concentrations to the squares of the diameters. Increasing the diameters of the circles at the end point by using highly concentrated antigen standards or by diluting the antiserum should result in a further increase in accuracy, if the circles do not become too faint for adequate measurement. [If necessary, precipitates can be enhanced by adding co-precipitating proteins or by staining with tannic acid or other agents (*8, 45*).] Increasing the number of antigen standards will increase the accuracy of the $c = d^2$ calibration line. The reliability of semilogarithmic plots is questionable.

Migration is more rapid in cellulose acetate membranes than in agar gel (*46, 47*). In collaborative studies (*44*) of this medium, we have obtained accurate end points over a wide range of diameters within 48 h for α_2-macroglobulin, a large and slowly diffusing antigen that takes much longer to equilibrate over a comparable range in gels. Such media could provide the basis for a commercial RID plate that is both rapid and reliable. This could be combined with a mechanized method for applying reagents and with an automated instrument for measuring and calculating results such as Johnson et al. describe.[2]

In the absence of such developments, it is likely that other methods will soon replace RID in routine clinical practice. Indeed, workers in Heremans' laboratory, where the most reliable RID technique was devised, have recently found (*48*) that a nephelometric automated immunoprecipitin system produces equivalent results in a far shorter time.

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