isoenzymes other than MM, or of enzymatically inactive forms, or both. This is more apt to occur in patients than in healthy subjects because disease states can be associated with increases in the amount of serum CK-MM and CK-BB and with release of inactive enzyme forms. These results open up the possibility that systematic studies of CK-MM by RIA will yield information about disease states that was not obtainable from analysis of data with enzyme assays.

This approach to measurement of serum enzyme amounts, then, is practical. Whether additional information about the patient can be obtained remains yet to be demonstrated. In our laboratory, specific patient groups are being investigated and we are collecting more data on healthy Negro subjects. Work is also in progress to shorten the assay so that it can be completed in one day.

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References

Appendix: The Assessment of Antibody Affinity from Radioimmunoassay

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Although the Scatchard plot (AI) remains the most used method of graphical analysis of affinity, radioimmunoassay data are frequently presented in the form of bound/free as a function of antigen concentration. I wish to show here that it is possible, in analogy with the methods of enzymology and other fields, to deduce association constants from such plots. If free antigen concentration is plotted on the abscissa, one can do so directly. However, if total antigen concentration is plotted instead, the value of the reciprocal of the association constant obtained must be corrected by the value of one half the antibody binding site concentration.

Let $K = \text{association constant (affinity)}$
$B = \text{concentration of bound complex}$
$F = \text{free antigen concentration}$
$A = \text{total antibody binding site concentration}$
$R = B/F$
$T = \text{total antigen concentration} = B + F$

Then, for a single antibody species interacting with

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antigen, the laws of equilibrium state that
Free antibody + free antigen
\[ (A - B) \xrightarrow{F} \text{antigen–antibody complex} \]
\[ \frac{K(A - B)}{B} \]
\[ K = \frac{B}{F(A - B)} \] (1)
or
\[ \frac{B}{F} = K(A - B) \] (2)

Equation (2) defines the classic linear Scatchard plot of \( B/F \) vs. \( B \), with slope equal to \(-K\). Now suppose the experimental design is to measure \( B/F \) as a function of increasing antigen concentration. Then the initial \( B/F \) is

\[ R_0 = \lim_{F \to 0} \frac{B}{F} = \lim_{B,F \to 0} K(A - B) = KA \] (3)

Then the midpoint of the titration curve, when \( B/F \) is half its initial value, is defined by

\[ K(A - B) = \frac{B}{F} = R = \frac{1}{2} R_0 = \frac{1}{2} KA \]

\[ KA - KB = \frac{1}{2} KA = 0 \]

\[ B = \frac{A}{2} \] (4)
i.e., this is the point when the concentration of bound complex is just half the total antibody concentration; i.e., half the antibody sites are occupied.

Substituting equation 4 into equation 2, we have

\[ \frac{A/2}{F} = K \left( A - \frac{A}{2} \right) \]

\[ \frac{1}{F} = K \text{ or } F = \frac{1}{K} \] (5)

Thus, when \( R \) reaches \( \frac{1}{2} R_0 \), \( F \) is exactly equal to \( 1/K \). This is completely rigorous for a single equilibrium (homogeneous antibody) (Figure A1). However, it must be emphasized that the value of \( F \) plotted on the abscissa must include all the free antigen, both labeled and unlabeled, and \( R_0 \) used in determining the midpoint must be obtained by extrapolating total free antigen, including tracer, to zero.\(^1\)

Now let us examine the problem with this transition point in a plot of \( B/F \) vs. \( T \). When \( R = \frac{1}{2} R_0 \), as we have shown, \( B = A/2 \) from equation 4, and \( F = 1/K \) from equation 5. Adding these, we have

\[ T = B + F = \frac{A}{2} + \frac{1}{K} \] (6)

\(^1\) If \( R_0 \) is less than \( KA \), for instance because tracer concentration is large so that antigen cannot be extrapolated to zero, then \( K \) will be underestimated by \( 1/F \) at the apparent midpoint, which will be too far to the right.

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Fig. A1. Schematic plot of \( B/F \) vs. \( F \) or \( T \) for a homogeneous antibody population

Curves are very similarly shaped, but the midpoint of the plot of \( B/F \) vs. \( T \) has a term dependent on antibody concentration, whereas the midpoint of \( B/F \) vs. \( F \) is exactly \( 1/K \), independent of antibody concentration.

Thus, the value of total antigen \( T \) at the transition point is a function of antibody concentration as well as \( K \). If \( A/2 \) is very small relative to \( 1/K \), the transition point is a reasonable approximation of \( K \). In many cases, however, the concentration of antibody used will be such that \( A/2 \) is comparable to or greater than \( 1/K \), in which case the curve for \( B/F \) vs. \( T \) will be shifted to the right relative to the curve for \( B/F \) vs. \( F \), and the value of \( T \) at the transition will reflect predominantly the antibody concentration, an underestimate of \( K \) (see Figure A1).

Finally let us consider the case of heterogeneous antibody binding a single antigen. Just as the Scatchard plot becomes more complex and less useful, so does this approach. Consider a antibody populations of binding site concentrations \( A_i \) and affinity \( K_i \). The amount of antigen bound by each is \( B_i \), so from equation 1,

\[ B_i = \frac{A_i K_i F}{1 + K_i F} \] (7)

and total bound

\[ B = \sum_i B_i = \sum_i \frac{A_i K_i F}{1 + K_i F} \] (8)

so that

\[ \frac{B}{F} = \sum_i \frac{A_i K_i}{1 + K_i F} \] (9)

and the total antibody concentration is \( A = \sum_i A_i \). Now

\[ R_0 = \lim_{B,F \to 0} \frac{B}{F} = \lim_{F \to 0} \frac{\sum_i A_i K_i}{1 + K_i F} = \sum_i A_i K_i \] (10)

Therefore our condition for the midpoint of the titration curve is

\[ \frac{B}{F} = R = \frac{1}{2} R_0 = \frac{1}{2} \sum_i A_i K_i \] (11)

Now suppose we define an average affinity \( K_{av} \) as a weighted average of the affinities, weighted in propor-
Fig. A2. Schematic Scatchard plot for homogeneous and heterogeneous antibodies

Homogeneous antibody produces a linear Scatchard plot with intercepts shown, where the midpoint on the $B/F$ axis corresponds to the midpoint on the $A$ axis. Heterogeneous antibodies produce a concave-up Scatchard plot (dashed curve) so that the point where $B = A/2$ does not correspond to the point where $B/F = 1/2 R_0$ on the curve. $K_0$ is the negative of the slope of the tangent to the curve at the point where $B = A/2$, and represents a median affinity (where half the antibody sites are occupied). $K_{av}$ the mean affinity, is the ratio of the intercepts $R_0/A$, i.e., the slope of the chord to the concentration of that antibody species. Then

$$K_{av} = \frac{1}{\sum_i A_i K_i} = \frac{1}{A} \sum_i A_i K_i$$

(12)

Therefore, dividing equation 11 by $A$ and substituting equation 12, we obtain

$$\left(\frac{1}{F}\right)\left(\frac{B}{A}\right) = \left(\frac{1}{A}\right) \sum_i A_i K_i = \frac{1}{2} K_{av}$$

$$\frac{1}{F} = K_{av} \left(\frac{A}{2B}\right)$$

(13)

Thus $1/F$ will equal $K_{av}$ if, and only if, $B = A/2$ at this point, which is exactly where $K_0$ is defined as the median affinity from a curved Scatchard plot (see Figure A2). Thus $1/F = K_{av}$ only if $K_{av} = K_0$.

It is easy to see graphically that $B/F = R_0/2$ when $B = A/2$ only if the Scatchard plot is linear, i.e., only if the antibody population is homogeneous (Figure A2). (On the other hand, $K_0$, the negative slope of the tangent at $B = A/2$, could under special conditions of symmetry equal $K_{av}$, even for a curved Scatchard plot.) However, for the general case of a curved Scatchard plot due to antibody heterogeneity, if the total antibody site concentration $A$ is known, the average affinity can be obtained by dividing $B/F$ at $R_0/2$ by $A/2$, i.e.,

$$K_{av} = \left(\frac{2}{A}\right) \left(\frac{B}{F}\right) \text{ when } \frac{B}{F} = \frac{R_0}{2}$$

(14)

or simply

$$K_{av} = \frac{R_0}{A}$$

(15)

It must be emphasized that for this result to apply, $R_0$ must be the true limit of $B/F$ as $F$ (and total) go to zero, and so can be obtained only if the labeled antigen (tracer) in a radioimmunoassay is infinitesimal or can be extrapolated to zero. The total antibody site concentration ($A$) can be determined from the plateau value of bound antigen at large antigen excess when antibody is saturated (for instance, by plotting $B$ vs. $F$).

Regardless of definitions of average affinities, if the affinities of the antibody subpopulations are sufficiently different from one another, one can in principle decompose a plot of $B/F$ vs. $F$ into a step function of subpopulations with intrinsic values of $K$. One can pick each affinity $1/K$ from the value of $F$ at the midpoint of each successive transition, in a way similar to that established for individual pK values in a complex pH titration.

The effect of antibody concentration on a plot of $B/F$ vs. total antigen has been derived independently elsewhere by a different approach (A2), but is still not widely appreciated. Moreover, as has now been demonstrated here, one can avoid these pitfalls altogether by simply plotting $B/F$ vs. free antigen rather than total antigen.

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