

tation of Immunoglobulins by Inhibition of Precipitation of ^{125}I Labelled Antigens.

For quantitative estimation of immunoglobulins, either in whole sera or isolated preparations, we have slightly modified a method originally described to us by Dr. John Fahey in which unlabelled antigen is introduced at varying dilutions in a reaction mixture containing a standard amount of antiserum at a limiting dilution and ^{125}I labelled antigen. The unlabelled antigen competes for the antibody and thus "inhibits" the precipitation of the labelled antigen. Comparison with a standard curve for inhibition by a solution containing a known amount of unlabelled antigen allows accurate quantitation of the unknown.

The inhibition assay is performed as follows (slightly modified from ref. 24):

As in direct precipitation, 50 μl of labelled antigen diluted in 3% BSA-tris such that 50 μl contain approximately 2×10^4 cpm, is added first. Next 5 μl of unlabelled test or standard antigen at varying dilutions in 3% BSA-tris is added and the tubes mixed. All tests are done in duplicate. Last, 50 μl of antiserum at a dilution (in S-dil) chosen such that the amount added is sufficient to precipitate about 2/3 of the maximum number of counts precipitable by that antiserum is added (See Fig. 1-). Since rapid mixing is essential, antiserum is generally added to two tubes at a time

and the unlabelled competitive (inhibitor) antigen simply competes for place in the precipitate, thereby decreasing the specific radioactivity of the total precipitated antigen. This decrease in specific activity is thus due only to dilution and is inversely proportional to the concentration of unlabelled antigen.

Put into mathematical terms, if for each assay tube:

P = fraction of radioactivity precipitated,

Ab = μg of antigen precipitated (bound) by the amount of antibody used,

Ag = μg of labelled antigen used,

Ag^* = number of counts per minute in Ag and

u = varying amount (in μg) of unlabelled (inhibitor) antigen added

then

$\frac{Ag^*}{Ag}$ = specific radioactivity of the labelled antigen preparation and

$\frac{Ag^*}{u + Ag}$ = specific radioactivity of total antigen in tube when varying amounts of unlabelled antigen are added.

When no inhibitor is present,

$$P = \frac{Ab \cdot \frac{Ag^*}{Ag}}{Ag^*} = \frac{Ab}{Ag} = \text{maximum fraction of counts precipitated in the assay}$$

When varying amounts of inhibitor (u) are present,

$$P = \frac{Ab \cdot \frac{Ag^*}{u + Ag}}{Ag^*} = \frac{Ab}{u + Ag}$$

and

$$\frac{1}{P} = \left(\frac{1}{Ab} \cdot u \right) + \frac{Ag}{Ab}$$

This is in the slope intercept form of the equation for a straight line when $1/P$ is plotted against u . The inverse of the ordinate intercept of this line is equal to Ab/Ag , the maximum fraction of labelled antigen which is precipitable by the

amount of antibody used. The slope, $1/Ab$, decreases as the absolute amount of antigen precipitated increases, hence as the amount of antibody used increases.

Data showing a typical reciprocal plot (linear) standard curve for an inhibition assay is presented in Fig. 2a. (For comparison, the same data with percentage of labelled antigen plotted directly as a function of amount of inhibitor is presented in Fig. 2b). Although the data in both figures are presented in terms of μg of inhibitor added, it is, of course, equally possible to use a standard of unknown inhibitor concentration, e.g., normal serum, and express values for unknowns as ul of standard per ul unknown.

The inhibition curve presented here (Fig. 2b) was chosen to show the most common departure from linearity observed in this system: a small initial lag before linearity is established. The "lag" may be avoided by dropping the antibody concentration or raising the amount of antigen in the labelled preparation, however, it is frequently more convenient just to ignore the early part of the curve and read values from the linear region only. In this case, the true y intercept, (i.e., the maximum fraction of labelled antigen precipitable at the antigen and antibody concentrations used in the assay) may be determined by extrapolation of the linear portion of the curve.

The observed "lag" is probably due to the total antigen concentration being below saturation at the antibody concentration chosen, since theoretically linearity only obtains when Ab (the amount of antigen precipitated in the assay) is constant. This interpretation is borne out by the demonstration that for a given amount of labelled antigen, the length of the "lag" increases with increasing antibody concentration and decreases with increasing antigen concentration.

Sensitivity of the assay varies according to the concentration of antibody and labelled antigen. Generally, the minimum amount of immunoglobulin carrying

a particular allotypic antigen detectable is in the neighborhood of 0.02 mg/ml serum, although if need be the lower limit can be decreased by as much as an order of magnitude. Determinations may be carried out without interference even in the presence of a 20,000-fold excess of a non-cross reacting immunoglobulin. Thus, as little as 10 μ g of allotype-carrying immunoglobulin may be detected in a mouse with roughly 30 mg of total immunoglobulin.

Two precautions are necessary to prevent serious errors in the quantitative determinations: It is important to

a) Avoid the introduction of a large amount of extraneous antigen-antibody complex, which will nonspecifically consume Clq and therefore prevent complete precipitation of the labelled complex (see earlier section of Clq), and

b) Avoid situations where the standard or the unknown differs from the labelled antigen and does not carry all of the antigenic specificities detected by the antiserum in the assay. This can occur with cross reacting antigens (such as allotypes) if the antiserum used reacts with two specificities, both of which are present on labelled antigen molecules but only one of which is present on the inhibitor. In such a case the inhibitor cannot inhibit precipitation completely, therefore, the reciprocal plot for the inhibitor is not linear, and quantitative comparisons become hazardous. If the standard and the labelled antigen are identical but the "unknown" is different, values for the unknown read from the standard curve are invalid!

Linearity for a cross-reacting standard may be obtained by subtracting the fraction of radioactivity precipitation not inhibitable from the total fraction precipitated before converting to the reciprocal. It is, however, more satisfactory to choose appropriate combinations of antiserum and labelled antigen or to absorb the antiserum first, so that such manipulations are unnecessary.