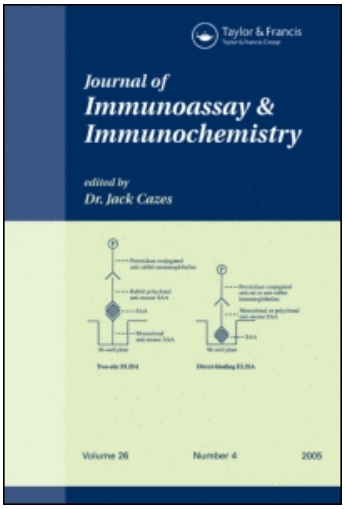

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A SENSITIVE DOUBLE ISOTOPE MODIFICATION OF THE FARR ASSAY USING BETA-PARTICLE EMITTERS

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ABSTRACT

A sensitive modification of the ammonium sulfate precipitation assay for antibody affinity is described. The assay combines small reaction volumes and a ^{32}P volume marker to determine the average relative affinity of small amounts of antibody to DNP hapten. The assay is relatively safe since two beta-particle emitters are utilized instead of two gamma-ray emitters. The assay has proven useful for the determination of small amounts of antibody such as might be found in secretions and column fractions.

KEYWORDS: Radioassay, Farr assay, DNP (dinitrophenyl), Affinity, Assay.

INTRODUCTION

The high sensitivity and relative ease with which the ammonium sulfate precipitation assay for antibody affinity (Farr assay; (1)) can be performed has given rise to its common use for the determination of affinity of antibody. The assay consists of the reaction of an antibody containing solution with a non-precipitable radiolabeled antigen at various

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concentrations. The antibody-antigen complexes, together with non-antibody immunoglobulins are then precipitated with ammonium sulfate. The radiolabeled antigen in the precipitate represents the amount of antigen bound and is used to determine the relative affinity constant of the antibody. The determination of the amount of antigen bound in an assay is usually performed indirectly by counting an aliquot of the supernatant of the reaction mixture after precipitation and noting the reduction in free antigen. This traditional method, however, is insensitive since small concentrations of antibody cause only a small percentage of the total free hapten to be bound. A more sensitive method of determining bound hapten would be to directly count the precipitate. However, direct counting of the precipitate is difficult due to the presence of unbound antigen. A possible solution, washing unbound antigen from the precipitate with an ammonium sulfate solution before counting, can cause a shift in equilibrium in monovalent antigen systems (2) resulting in inaccurate affinity determinations and is therefore unsatisfactory. Gaze and coworkers (3) utilized a double isotope system which consisted of an ^{125}I-labelled antigen and a ^{22}Na volume marker which allowed direct counting of the precipitate by subtraction of unbound antigen which was represented by the volume marker. Unfortunately, this system employs two relatively dangerous gamma-ray emitters, ^{125}I and ^{22}Na, which have to be handled with considerable caution and which necessitates the use of a

gamma counter. We have developed a double isotope modification of the Farr assay that utilizes two beta-particle emitters, ^{3}H and ^{32}P, which are safer, easier to handle and can be counted in a liquid scintillation spectrometer. This assay modification allows direct counting of precipitates, providing an easy and highly sensitive means (antibody concentration = 1 µg/ml) of determining relative antibody affinity.

MATERIALS AND METHODS

Radiochemicals

^{3}H-dinitrophenyl-L-lysine (3.6 mCi/mM) was obtained from New England Nuclear. ^{32}P-disodium phosphate (116 mCi/mM) was obtained from ICN Chemical and Radioisotope Division.

Assay Method

100 µl of either antibody solution or a similar solution not containing antibody is added to Kimax centrifuge tubes (2 ml). 10 µl of a one-third dilution of germ-free rat serum (GFRS) or 10 µl of PBS (pH 7.5) is added to the tubes followed by 25 µl of ^{32}P disodium phosphate in PBS to give approximately 12,000 cpm/tube. 25 µl of various concentrations of ^{3}H-DNP-L-lysine in PBS is then added to the tubes and the tubes are vortexed and then incubated in a 37°C shaking water bath for 2 hrs. The ^{3}H-DNP-L-lysine is added at concentrations of 1.2 x 10^{7} to 3.3 x 10^{6}M to give approximately 10,000 cpm/tube. These concentrations of ^{3}H-DNP-L-lysine provide a highly

TABLE 1

CONTENTS OF REACTION TUBES

Tube	Non-Antibody (NAb-100 µl) Antibody (AB-100 µl)	1/3 GFRS (10 µl)[a]	^{32}P-dsp (25 µl)[b]	^{3}H-DNP-L-lysine (25 µl)
1-3	NAb	+	+	$1.2x10^{-7}$M
4-6	NAb	+	+	$3.7x10^{-7}$M
7-9	NAb	+	+	$1.1x10^{-6}$M
10-12	NAb	+	+	$3.3x10^{-6}$M
13-15	Ab	+	+	$1.2x10^{-7}$M
16-18	Ab	+	+	$3.7x10^{-7}$M
19-21	Ab	+	+	$1.1x10^{-6}$M
22-24	Ab	+	+	$3.3x10^{-6}$M
25-27	NAb	-	+	$1.2x10^{-7}$M
28-30	NAb	-	+	$3.7x10^{-7}$M
31-33	NAb	-	+	$1.1x20^{-6}$M
34-36	NAb	-	+	$3.3x10^{-6}$M

a) Germ Free Rat Serum

b) ^{32}P-disodium phosphate

sensitive assay capable of determinations of the relative affinity of antibody at concentrations in the 1.0 µg/ml range. Concentrations of antigen may be increased to determine the relative affinity constant of higher concentrations of antibody. The contents of the individual reaction tubes are described below:

All tubes are prepared in triplicate. After the 2 hour incubation, 150 µl of saturated $(NH_4)_2SO_4$ is added to each of the tubes, and the tubes are vortexed thoroughly and incubated

overnight at 4°C. After the incubation, tubes #1 through #24 are centrifuged at 1000 xg for 10 minutes. Tubes #25-36 are not centrifuged since there is no precipitate and since aliquots from these tubes are used to determine the originally added dpm. 50 µl of supernatant from all tubes are carefully removed and added to the vials followed by 4 ml of scintillation fluid (Aqua-Sol, New England Nuclear; see discussion). The vials are vortexed thoroughly. The remaining supernatant is carefully removed from reaction tubes #1 through #24 without disturbing the precipitate and is discarded. Distilled H_2O (125 µl) is added to the remaining precipitate in tubes #1 through #24 and the tubes are vortexed thoroughly to dissolve the precipitate. The dissolved precipitates are removed with a Pasteur pipette and placed in scintillation vials. Each reaction tube is washed with 1.0 ml of distilled H_2O and the wash placed in the respective scintillation vial using the same Pasteur pipette as was used to originally remove the precipitate. This washes the reaction tube as well as the pipette. The scintillation vials are vortexed again and are counted on two channels by a liquid scintillation spectrometer for 10 minutes so that all vials containing the same concentration of DNP-L-lysine are counted together. The counting channels are adjusted so that one channel counts the ^{3}H spectrum and one channel counts the higher energy ^{32}P spectrum but excludes the lower energy ^{3}H counts.

Calculations

The calculation of the relative average affinity constant

(K_o) for an antibody source can be performed with a knowledge of the amount of antigen bound by a fixed amount of antibody at various antigen concentrations. In order to determine the amount of ^3H labeled antigen that is bound in our assay, it is necessary to measure the ^3H in the precipitate. However, without washing the precipitate, the free antigen in the solution around the precipitate will contribute to any determination of the ^3H which is bound. Therefore, we have included a volume marker, ^{32}P-disodium phosphate, (Table 1) which allows subtraction of unbound antigen from bound antigen.

^3H dpm is excluded from the ^{32}P channel by the setting of the counting window so that low energy ^3H is excluded thus allowing ^{32}P dpm to be counted and read directly. The counting of ^3H dpm is more difficult, because some lower energy ^{32}P dpm will be counted in the low energy ^3H window. At our window settings, on a Beckman LS-100C spectrometer, we have found that the ^{32}P dpm counted in the ^3H window remains at a constant 3% of the ^{32}P dpm counted in the ^{32}P window and that this 3% remains constant in spite of any slight variations in quench of the smaples. The lack of variation in the 3% figure when there are small changes in the external standard ratio appears to be because ^{32}P is a high energy beta particle emitter, and therefore, undergoes minimal shifting of its energy window at various degrees of quenching. Therefore, 3% of ^{32}P dpm counted on the ^{32}P channel is subtracted from ^3H dpm counted in the ^3H window to arrive at true ^3H dpm of the counted samples.

If we assume:

Psn = ^{32}P dpm in 50 µl of supernatant from tube containing antigen concentration X and no antibody.

Ppn = ^{32}P dpm in precipitate from tube containing antigen concentration X and no antibody.

Hsn = ^{3}H dpm in 50 µl of supernatant from tube containing antigen concentration X and no antibody.

Hpn = ^{3}H dpm in precipitate from tube containing antigen concentration X and no antibody.

Psa = ^{32}P dpm in 50 µl supernatant from tube containing antigen concentration X and antibody.

Ppa = ^{32}P dpm in precipitate from tube containing antigen concentration X and antibody.

Hsa = ^{3}H dpm in 50 µl of supernatant from tube containing antigen concentration X and antibody.

Hpa = ^{3}H dpm in precipitate from tube containing antigen concentration X and antibody.

Hc = ^{3}H dpm in 50 µl from tube containing antigen concentration X, no antibody, and no carrier protein.

then ^{3}H dpm bound in the precipitate can be calculated:

^{3}H dpm bound = bound ^{3}H dpm in ppt. - free ^{3}H dpm in ppt.

or $$^{3}H \text{ dpm bound} - Hpa - \frac{Hsa \cdot Ppa}{Psa} - Hpn + \frac{Hsn \cdot Ppn}{Psn}$$

Using ^{3}H dpm bound we can then determine the concentration of bound antigen (b) in a reaction tube with DNP-L-lysine concentration X:

$$b = \frac{^{3}H \text{ dpm bound} \cdot X}{6.2 \cdot Hc}$$

and the concentration of free antigen (c) in a reaction tube with DNP-L-lysine concentration X:

$$c = X - b$$

The same calculations are performed for all antigen concentrations used in the assay to arrive at a b and c for each antigen concentration.

The K_o is defined as the reciprocal of c at which 50% of the total available antibody binding sites are occupied by antigen (1). The Langmuir plot of 1/r vs. 1/c, where r = the total number of available antibody binding sites, may be used to determine the total number of antibody binding sites available and may, therefore, be used to determine the average K_o of the antibody. In the case of a monovalent hapten, the relation for the Langmuir isotherm is established from the equation:

$$\frac{1}{r} = \frac{1}{ncK_o} + \frac{1}{n}$$

where n equals the number of antibody sites bond to antigen (4). We may therefore, in a monovalent system, plot 1/b vs. 1/c to arrive at the 1/c at which 50% of the total available antibody binding are occupied, as shown in Figure 1 below.

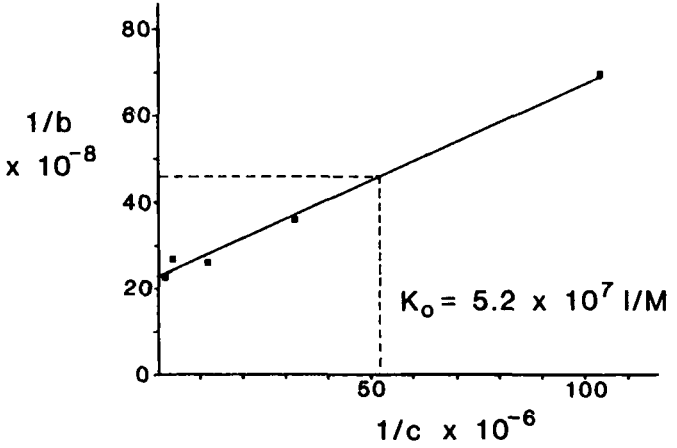

FIGURE 1. Double Reciprocal Plot of 1/b vs 1/c on a fraction of rat milk enriched in secretory IgA antibody (7). Extrapolation of line to y axis yields total available binding sites (4.4×10^{-10} M/l). Extrapolation to the x axis at one-half that number (2.2×10^{-10} M/l) will provide a determination of the K_o of the antibody ($K_o = 5.2 \times 10^7$ l/M).

DISCUSSION

The modification of the Farr ammonium sulfate precipitation assay described here utilized a ^{32}p volume marker and a ^{3}H labeled antigen. The assay is a rapid and sensitive means of determining the relative affinity of antibody in a solution. We selected ^{3}H as a label for the antigen, DNP-L-lysine could be readily purchased and is relatively safe to handle, requiring no shielding. The energy (0.018 MeV maximum) was also sufficiently low to allow separation of counts from those of a high energy beta particle emitter (1.71 MeV maximum), was selected as a volume marker because it allowed adequate differentiation from ^{3}H counts, was easily shielded by plastic or glass, being a charged particle emitter, and could be readily purchased as ^{32}P-disodium phosphate, a component of the PBS buffer used in the ssay. Additionally, ^{32}P dpm counted in the ^{3}H counting channel could be easily accounted for since the window was not shifted by quenching the sample. Any potential problem created by non-specific binding of ^{32}P to protein was eliminated by inclusion of non-antibody containing protein control in each assay.

The calculations for the described assay were performed based on an assumption that the final reaction volume was that of the solution after addition of the ammonium sulfate. This was because of the reported inability of addition of ammonium sulfate to stop the antibody-antigen reaction in a monovalent system (2) thereby allowing development of an equilibrium based upon a new total volume (6). In view of the continued reaction of antigen with antibody after addition of the

ammonium sulfate, (2) sufficient time must be allowed for incubation in order to ensure equilibrium. We have allowed overnight incubation at 4°C which was considerably more time than the 20 minutes found to be satisfactory by Seppala (2).

^{32}P is an isotope with a relatively short half-life (14.3 days) and, therefore, scintillation vials that are being counted and used in a single calculation are counted sequentially to minimize any effect of ^{32}P half-life. The samples were counted for 10 minutes and the maximum error in counting attributable to ^{32}P isotope decay was determined as approximately 0.007%, a negligible amount of error in this assay.

Distilled water is added to the scintillation vials in our assay before counting in order to solubilize the antigen/salt solution fully and place the cocktail into "gel-phase" at room temperature and at 4°C. The amount of water needed was determined after consultation with the manufacturer of the scintillation fluid (New England Nuclear). All of our vials are counted in the "gel-phase" at 4°C.

In summary, we have developed a very sensitive modification of the Farr assay which can be used to determine the relative affinity of small concentrations of antibody in solution. We have previously utilized the examination of the kinetics and inhibition of haptenated bacteriophage neutralization to determine the relative avidity of antibody to DNP in rat milk (7). The current modification of the Farr assay provides a means of examining the actual affinity of the antibody binding

site for hapten when there are small amounts of antibody. We have found that the assay can be used to determine the affinity of antibody of various isotypes (IgG, IgA) at concentrations as low as 1 μg/ml.

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