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QUANTITATION OF INTERACTING MOLECULAR SPECIES AND MEASUREMENT OF MOLECULAR AVIDITY BY SINGLE RADIAL (IMMUNO) DIFFUSION

Akindele O. Johnson
Che-Hung Lee

Naval Medical Research
and Development Command
Bethesda, Maryland 20889-5055

Department of the Navy
Naval Medical Command
Washington, DC 20372-5210

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The single radial diffusion (SRD) assay was originally developed by Mancini et al. for the quantitation of antigen or antibody. In the present paper, this method was applied to the study of any molecular interacting system involving the formation of a precipitate. The binding component of the system may or may not be an antibody, and can be a solubilized aggregate. The use of the number of moles or equivalences instead of the mass units to represent the amount of the interacting components was also introduced to yield information on molar or equivalent basis. The SRD method was used to study endotoxins (or lipid A) and their interactions with a broad spectrum of agents, e.g. new methylene blue, an anti-lipopolysaccharide factor from horseshoe crab, and monoclonal antibodies against lipid A. Molecules such as dextran sulfate, heparin and succinylated bovine serum albumin carrying multiple anionic groups similar to endotoxin aggregates, also demonstrated clear SRD phenomena. This further substantiated the applicability of the SRD method to the studies of interacting molecules involved with precipitate formation. The total content of the LPS-binding species in samples (e.g. serum, etc.) can be estimated in normality units respective to the molecule used as a standard.

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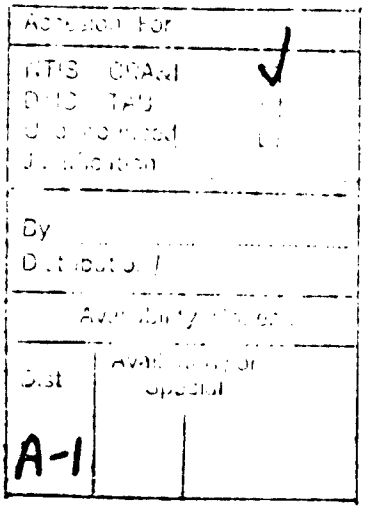

INTRODUCTION

Mancini et al. (1965) developed a single radial immunodiffusion (SRID) method for the quantitation of antigens. The method was further developed for studies related to antigens and antibodies (Vaerman et al., 1969a; 1969b; Mancini et al., 1970; Nash et al., 1970). It was realized that the positions of the antigen and the antibody in the SRID method could be switched. The modified method was used to quantitate the antibody instead of the antigen and was termed reverse single radial immunodiffusion (rSRID). The SRID and rSRID methods employed the phenomenon of the precipitate formation by antigen-antibody complex. Tan-Wilson et al (1983) applied this method to study the relative avidity of the antigen-antibody interactions. In all these papers, the method was limited to antibody-antigen interactions. Also, the quantities of the interacting components were expressed in mass units, which are proportional to the molecular weight of the component, greatly hampering the applicability of this method. In this paper, we extend this method to the quantitation of the components in interacting systems with precipitate formation. We also introduce the use of moles or equivalences for expressing amounts of the interacting components so that measurement of the quantities and the relative avidity of the components in these systems can be carried out with results in molar or equivalent scale. The interacting systems of new methylene blue with lipid A, LPS, dextran sulfate, heparin and succinylated

ABBREVIATIONS: SRID, single radial immunodiffusion; rSRID, reverse single radial diffusion; SRD, Single radial diffusion; LPS, lipopolysaccharide; ALF, anti-LPS factor; TEA, triethylamine; PB, phosphate buffer; SuBSA, succinylated bovine serum albumin; mAb, monoclonal antibody.

BSA were presented, based on the results from a double (immuno)diffusion assay (Johnson and Lee, 1989) that new methylene blue can bind to lipid A, lipopolysaccharides (LPS) and other polyanionic molecules. Other systems such as the anti-LPS factor (ALF; Muta et al., 1987) from horseshoe crabs with lipid A, and monoclonal antibodies with their antigen lipid A were also examined. A comparison among the relative avidities of the interacting components in these systems was conducted.

MATERIALS AND METHODS

Smooth LPS (s-LPS) of *E. coli* O111:B4 and O26:B6 were purchased from Sigma Chemical Company. Lipid A was prepared from the s-LPS of O111:B4 by hydrolysis in 2% acetic acid for two hours in a boiling water bath followed by washing with distilled water and lyophilization (Westphal et al., 1954; Grollman et al., 1964). Stock solutions (or suspension) of LPS or lipid A (2 mg/ml) were prepared by mixing LPS or lipid A with water containing 0.05% triethylamine (TEA). Other dilutions were prepared by mixing the stock solution with water or phosphate buffer (PB) containing 0, 0.02, 0.2 or 2% TEA. SuBSA was prepared by succinylation of bovine serum albumin according to Koltz (1967). The anti-LPS factor (ALF, 1.8 mg/ml in buffer) from horseshoe crab was kindly provided by Cape Cod Associates, Mass. New methylene blue, dextran sulfate and heparin were purchased from Kodak Chemicals, Pharmacia and Fisher, respectively. Protein A-agarose, ImmunoPure binding buffer and ImmunoPure elution buffer for affinity chromatography, and the BCA protein assay reagents were from Pierce.

Monoclonal antibodies against lipid A

Murine monoclonal antibodies (mAb: A78S1, A502, A506 and A549; of isotype IgG_{2b}) against lipid A from *E. coli* O111:B4 were generated by the methods of Kohler and Milstein (1975) and reported elsewhere (Lee, C.-H., 1987). The mAb was purified by protein A affinity chromatography. Their protein concentrations were determined by the BCA protein assay according to the protocol provided by the manufacturer, using purified myeloma proteins (Sigma Chemical Company) as standard.

Single radial immunodiffusion (SRID) or single radial diffusion (SRD)

For the SRD studies of the two compounds A and B, the diffusion plates were prepared as follows. Prepare 1% agarose (boiled and then cooled to 55°C) in 5 mM phosphate buffer (PB), pH 7.5 and 2.5 mM EDTA. Mix one volume of this solution with one volume of a second solution containing $2xG_B$ grams per ml of component B (LPS, lipid A, dextran sulfate, heparin or SuBSA) at 55°C in 5 mM PB, pH 7.5 and 2.5 mM EDTA. 8.7 ml of the mixture was poured into a leveled petri dish (8.6 cm diameter) to form an agarose gel bed of 1.5 mm thickness. Wells of 2.8 mm diameter were then cut into the gel bed and filled with 4 ul of component A (containing total amount of G_A grams). The gel plates were incubated at 4, 25, or 36°C in a sealed moist chamber for 48 hr or longer. The samples were scored as to the presence or absence of a precipitate ring formed around the well after intensification in 2-4% tannic acid for 10 min followed by washing three times with deionized H_2O. In experiments with methylene blue, the precipitate rings were observed directly by the color of the dye. The diameters of the ring (d) and the well (d_0) were measured. The area S of the precipitate ring, equal to $\pi(d^2 - d_0^2)/4$, was plotted against the amount of component A, G_A or G_A/mwt_A (in number of moles, where mwt_A is the molecular weight of component A) loaded into the well, yielding a linear curve. The slopes $\Delta S/\Delta G_A$ or $\Delta S/(\Delta G_A/mwt_A)$ of these graphs from experiments utilizing different component B concentrations in agarose gel were plotted against the inverse of [component B], i.e. $(G_B/ml)^{-1}$ or $(G_B/mwt_B/ml)^{-1}$, yielding a linear curve with a slope of $K = \Delta S \times \Delta G_B/\Delta G_A$ or $K' = \Delta S \times (\Delta G_B/\Delta G_A) \times (mwt_A/mwt_B)$ listed in Table I. The graphs shown in this paper were plotted in mass units, which can be converted into number of moles (or equivalences) through the respective molecular (or equivalent) weight of the interacting

Table I. Relative avidity of various interacting systems determined by single radial (immuno)diffusion.

Interacting systems					Relative avidity	
Component A		Component B				
Compound	Mol. weight	Compound	Mol. weight		Slope K $(mm)^{-1}$	Slope K' $(mm)^{-1}$
mAb A78S1	155,000	lipid A	1,750		0.0079	0.696
mAb A502	155,000	lipid A	1,750		0.0196	1.739
mAb A506	155,000	lipid A	1,750		0.0061	0.542
mAb A549	155,000	lipid A	1,750		0.0044	0.390
ALF	12,000	lipid A	1,750		0.0726	0.498
NMB[b]	360	lipid A	1,750	(inner ring)	0.258	0.0523
				(outer ring)	0.377	0.0778
NMB	360	dextran sulfate	500,000		0.0927	6.7×10^{-5}
NMB	360	heparin	16,000		0.0942	0.0021
NMB	360	SuBSA	68,000		0.284	0.0015

a. The avidity is expressed as slopes K and K', where $K' = K \times (mwt_A/mwt_B)$.

b. NMB stands for new methylene blue.

components as denoted in each Figure legend.

RESULTS AND DISCUSSION

Reversed single radial immunodiffusion (rSRID) of monoclonal antibodies with antigen lipid A

The rSRID of four monoclonal antibodies (A78S1, A502, A506 and A549) was carried out with various concentrations of antigen lipid A in the agarose gel. A linear relationship exists between the area S of the precipitin ring and the amount of antibody (G_A or G_A/mwt_A) loaded into the well (Figure 1a for A78S1; 1b for A502; data for the other antibodies, not shown). The slopes $\Delta S/\Delta G_A$ or $\Delta S/(\Delta G_A/mwt_A)$ of these curves decrease as the concentration of lipid A increases in the agarose gel. A clearer relation is shown in the plot of these slopes vs. $[\text{lipid A}]^{-1}$ for each antibody (Figure 1c). All the curves appear to be linear with respective slope $K = \Delta S \times \Delta G_B/\Delta G_A$ or $K' = \Delta S \times (\Delta G_B/\Delta G_A) \times (mwt_A/mwt_B)$ shown in Table I. The curves of A78S1, A506 and A549 pass through the origin, while that of A502 does not (Figures 1a, 1b and 1c).

SRD of interacting systems: new methylene blue with lipid A, anti-LPS factor (ALF) from horseshoe crabs with lipid A, and new methylene blue with dextran sulfate, heparin and SuBSA

All these systems under study exhibit a linear relationship between S and G_A or G_A/mwt_A. Some of the plotted curves are shown in Figures 2a and 2b. The graphs of $\Delta S/\Delta G_A$ or $\Delta S/(\Delta G_A/mwt_A)$ vs. $(G_B/ml)^{-1}$ or $(G_B/mwt_B/ml)^{-1}$ for each system (Figures 3a, 3b and 3c) appear to be linear with respective slopes $K = \Delta S \times \Delta G_B/\Delta G_A$ or $K' = \Delta S \times (\Delta G_B/\Delta G_A) \times (mwt_A/mwt_B)$ as listed in Table I. In the case of new methylene blue with lipid A, two precipitate rings (i.e. the inner ring, heavier in color, and the outer ring) were observed for each sample well. It is apparent that two different precipitates are formed with that of

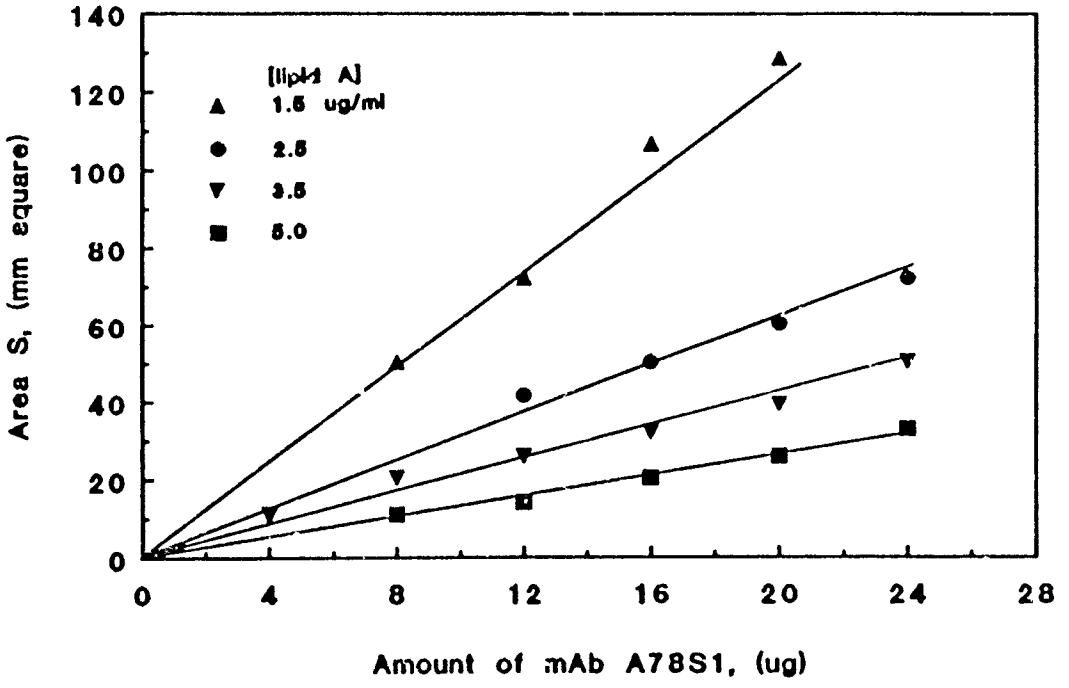
[Iipid A]
1.5 ug/ml
2.5
3.5
5.0
Area S, (mm square)
Amount of mAb A78S1, (ug)
0
20
40
60
80
100
120
140
0
4
8
12
16
20
24
28

FIGURE 1-a

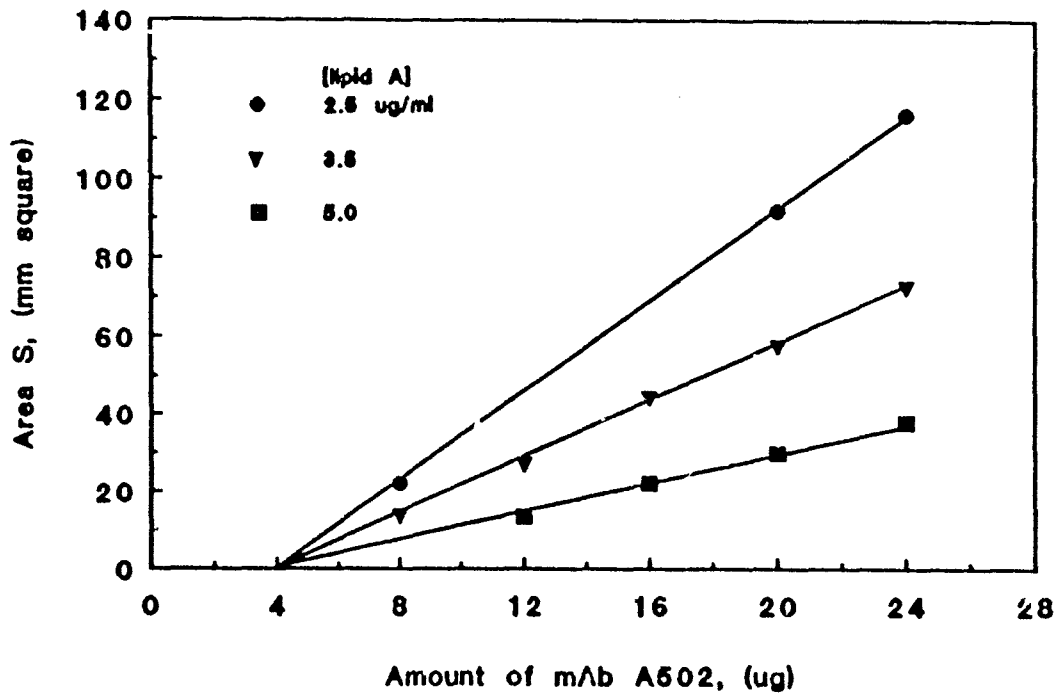
140
120
100
80
60
40
20
0
Area S, (mm square)
[Lipid A]
2.5 ug/ml
3.5
5.0
0
4
8
12
16
20
24
28
Amount of mAb A502, (ug)

FIGURE 1-b

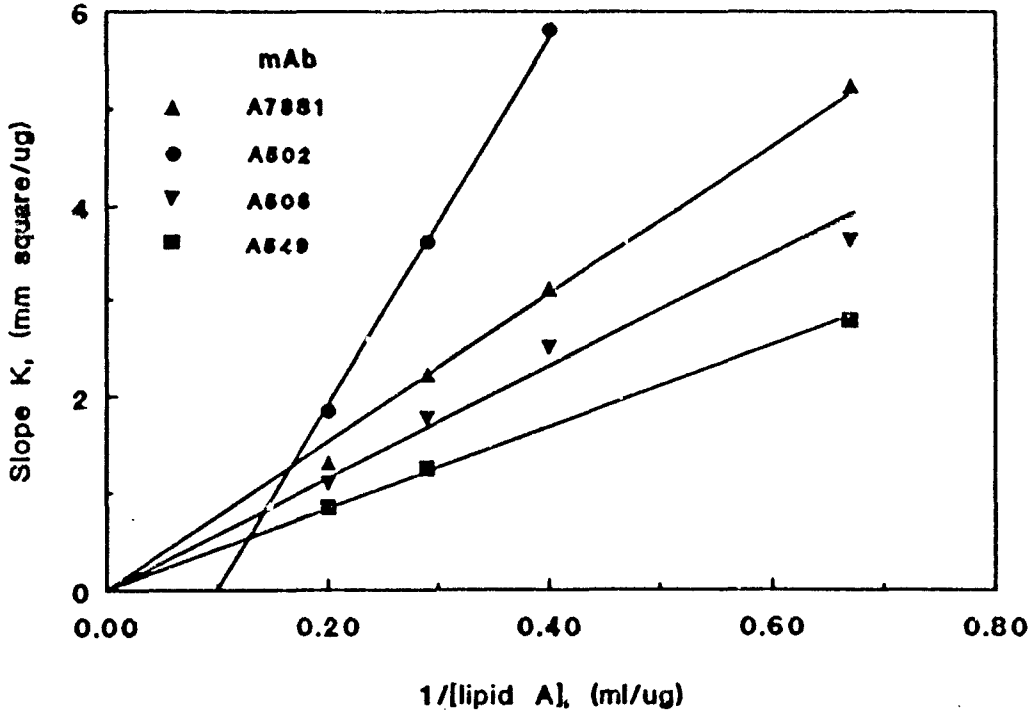
mAb
A7881
A502
A606
A549
Slope K, (mm square/ug)
1/[lipid A], (ml/ug)
0
2
4
6
0.00
0.20
0.40
0.60
0.80

FIGURE 1-c

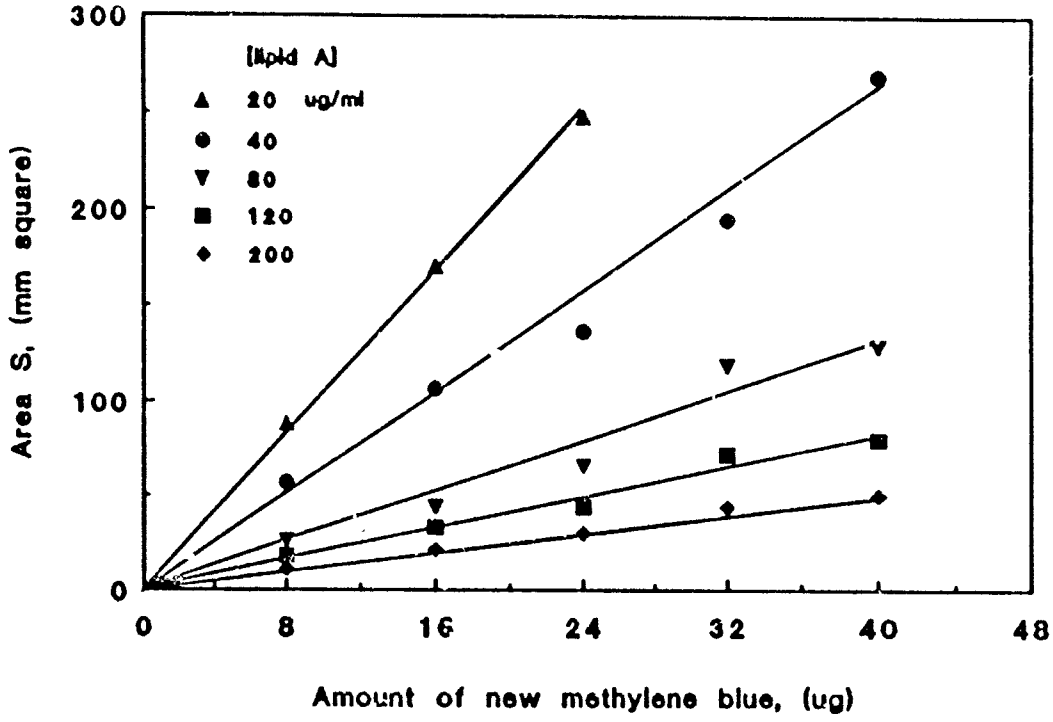
[lipid A]
20 ug/ml
40
80
120
200
Area S, (mm square)
300
200
100
0
0
8
16
24
32
40
48
Amount of new methylene blue, (ug)

FIGURE 2-a

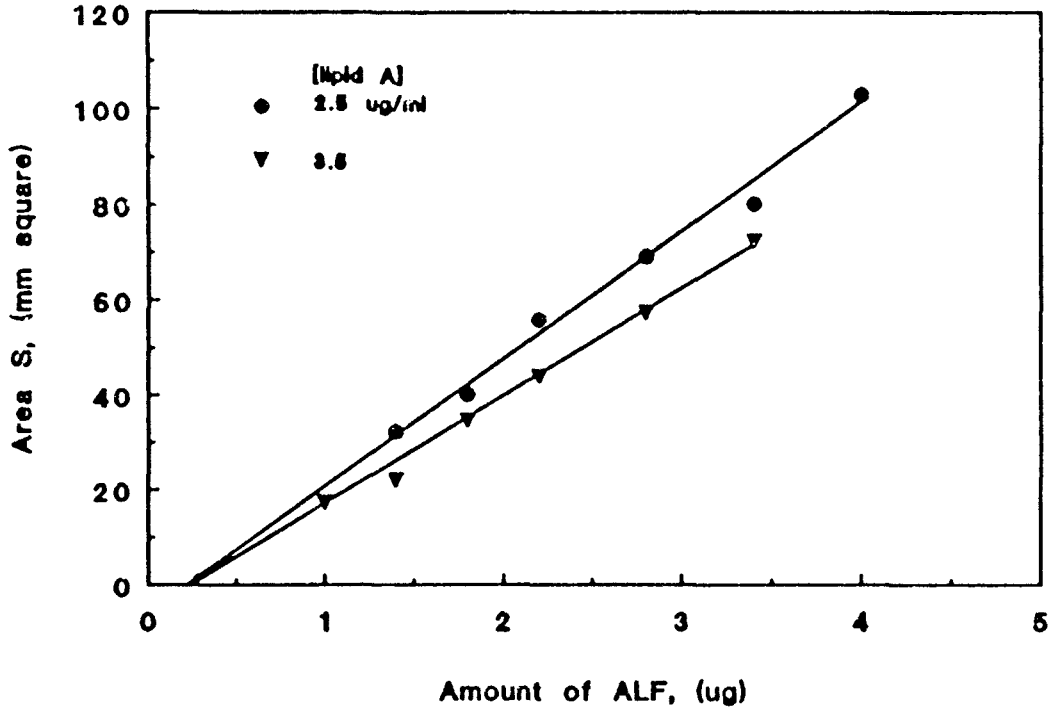
120
100
80
60
40
20
0
0
1
2
3
4
5
[Hpld A]
2.5 ug/ml
3.5
Area S, (mm square)
Amount of ALF, (ug)

FIGURE 2-b

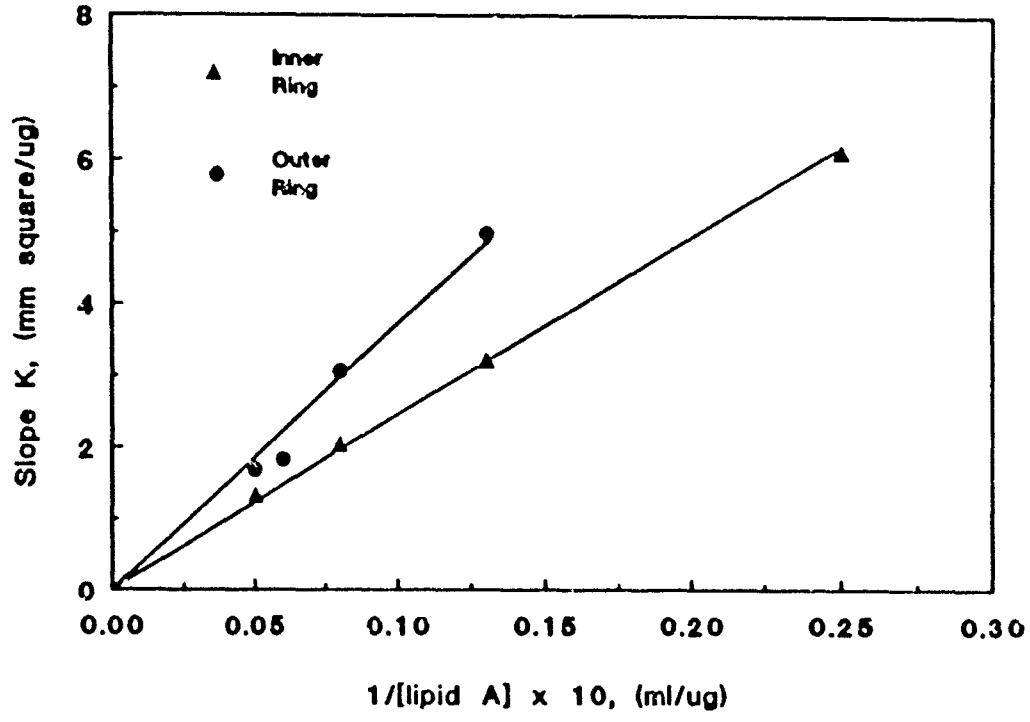
Inner Ring
Outer Ring
Slope K, (mm square/ug)
8
6
4
2
0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
1/[lipid A] x 10, (ml/ug)

FIGURE 3-a

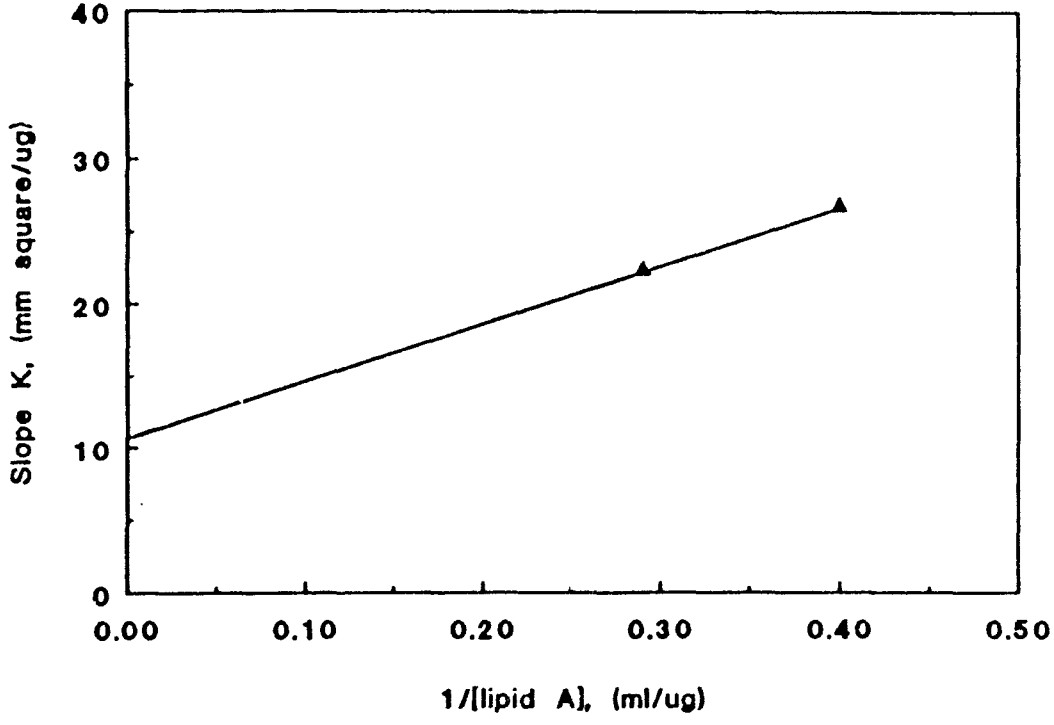
40
30
20
10
0
Slope K, (mm square/ug)
0.00
0.10
0.20
0.30
0.40
0.50
1/[lipid A], (ml/ug)

FIGURE 3-b

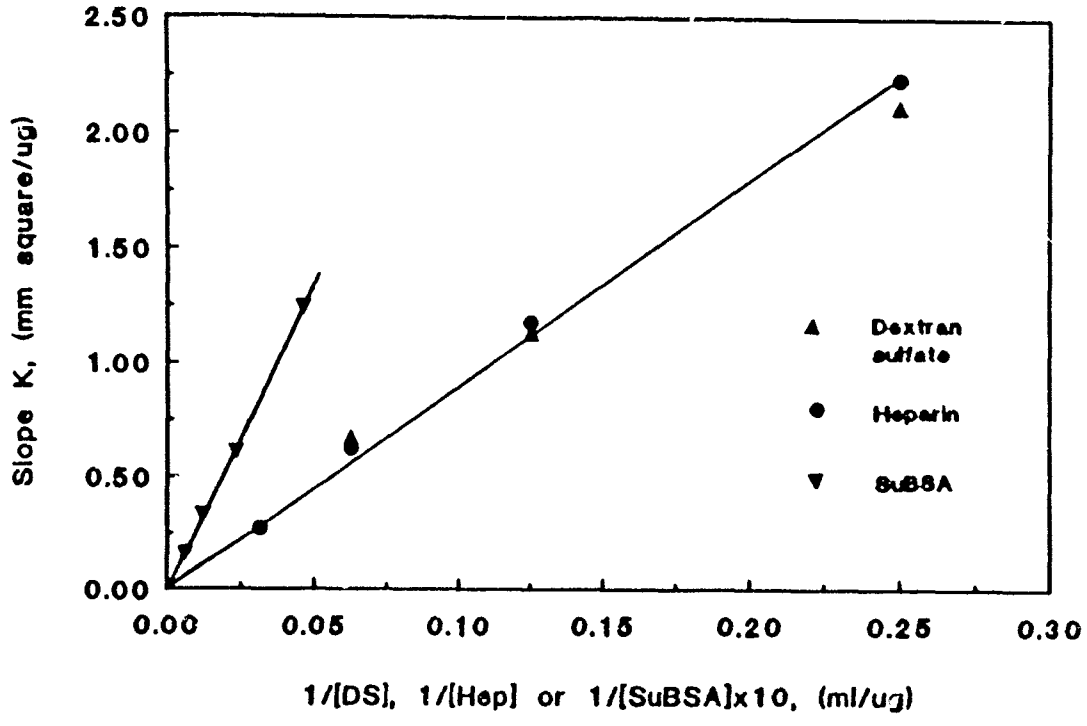
2.50
2.00
1.50
1.00
0.50
0.00
Slope K, (mm square/ug)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
1/[DS], 1/[Hep] or 1/[SuBSA]x10, (ml/ug)
Dextran sulfate
Heparin
SuBSA

FIGURE 3-c

the inner ring containing more new methylene blue per lipid A aggregate than that of the outer ring.

Relative avidity of the interacting components

The relative avidity of the components in an interacting system is represented by its slope, $K = \Delta S \times \Delta G_B/\Delta G_A$ or $K' = \Delta S \times (\Delta G_B/\Delta G_A) \times (mwt_A/mwt_B)$, listed in Table I. The value of K' reveals the avidity of the system on the molar basis, and allows comparison among systems. Larger K' value indicates higher avidity for component A (the diffusing component loaded in the well) and lower avidity for component B (the stationary component incorporated in the agarose gel). Thus, according to the magnitude of the K' values of different systems (Table I), the order of the relative avidity to lipid A for components A under study is A502 > A78S1 > A506 > ALF > A549 > new methylene blue; and the relative avidity to new methylene blue for components B is dextran sulfate > SuBSA > heparin > lipid A.

Effects of temperature and triethylamine on the avidity of new methylene blue-LPS system

Figures 4a and 4b show that increase of the concentration of triethylamine (TEA) or temperature causes a decrease in the slope $\Delta S/\Delta G_A$ of new methylene blue with a fixed endotoxin concentration in the agarose gel. This suggests that the avidity of the endotoxin (in the gel) increases, and/or that of new methylene blue (loaded in the well) decreases, presumably due to induced structural or conformational changes of the endotoxin aggregates or the dye-endotoxin complexes.

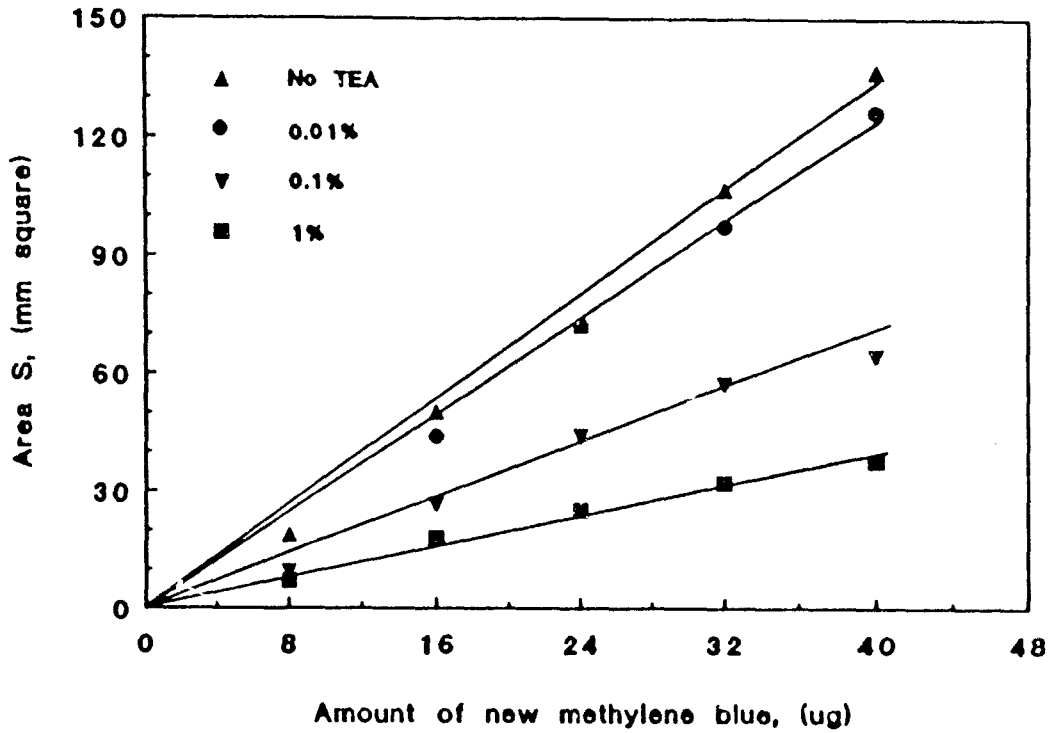
150
120
90
60
30
0
Area S, (mm square)
No TEA
0.01%
0.1%
1%
0
8
16
24
32
40
48
Amount of new methylene blue, (ug)

FIGURE 4-a

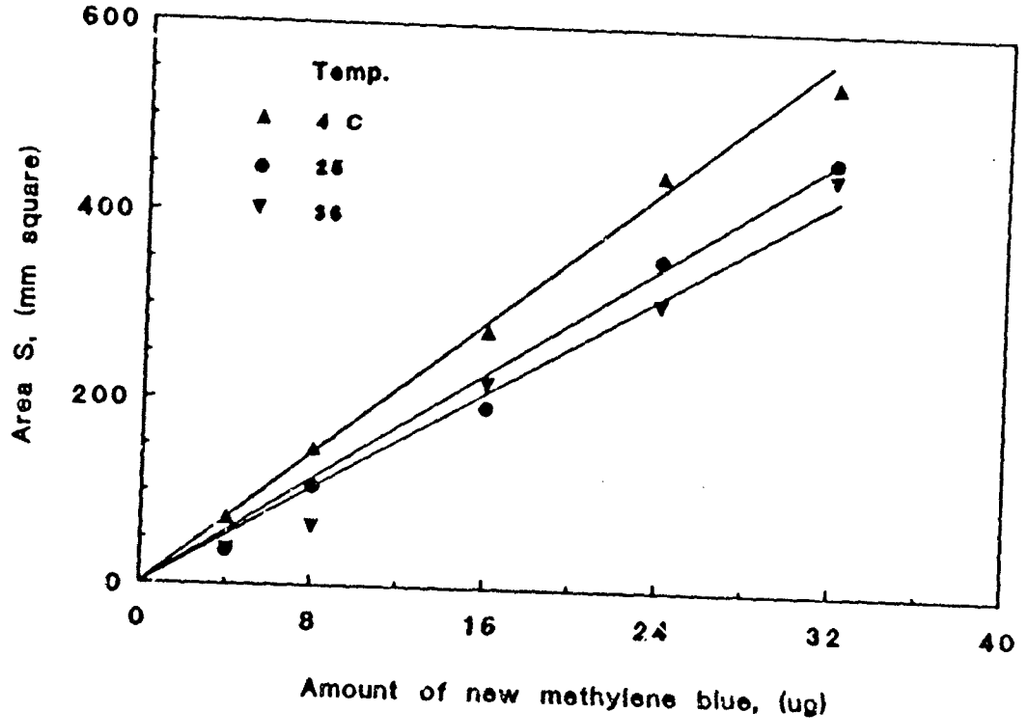
600
400
200
0
Area S, (mm square)
Temp.
4 C
25
36
0
8
16
24
32
40
Amount of new methylene blue, (ug)

FIGURE 4-b

Precipitability of complexes in SRD system

The phenomenon that the linear curve of the SRD data does not pass through the origin was observed previously (Vaerman et al, 1969a; 1969b; Tan-Wilson et al, 1983). It is speculated that the intercept of the linear curve of the S vs. G_A with either ordinate or abscissa or both (i.e. passing through the origin) may reveal the effect of components A on the precipitability of the complex formed in the interacting system (Figures 1a, 1b, 2a and 2b), even though such results may be introduced by experimental errors in many steps. If a curve intercepts with the abscissa, it indicates that precipitation would not occur unless the amount of component A loaded into the well is larger than the amount indicated at the intercept (Figure 1b for antibody A502, and Figure 2b for ALF). If it intercepts with the ordinate, it suggests that precipitation occurs even in the presence of trace amounts of component A.

For the graph of $\Delta S/\Delta G_A$ vs. $(G_B/ml)^{-1}$, the deviation of the intercept from the origin (Figure 1c, A502; Figure 3b) has no other meaning than just an indication of the theoretical maximal component B concentration in the agarose gel.

CONCLUSION

Our results have shown that single radial (immuno)diffusion can be used for quantitation of the components in any molecular interacting system which form visible precipitates. The measurement can be conducted with a standard molecule different from the unknown sample if the amount of the standard molecule is expressed in number of mole or equivalence. For example, the SRD method can be used for measurement of LPS-binding species using a known standard, since LPS binds to a variety of molecules and forms a precipitate. Thus, it should be possible to estimate the total normality of the precipitable LPS-binding species (Tesh et al., 1988) contained in serum or other samples, in respect to a standard (e.g. lipid A). In addition, the SRD method may allow one to look into the relative avidity of the interacting components and the precipitability of the complex formed by them.

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FIGURE LEGENDS

Figure 1. The SRD data for mAb A78S1 (a) and A502 (b) with lipid A in agarose gel, and the relation between the slope of the SRD data and 1/[lipid A] for mAb A78S1, A502, A506 and A549 (c). The scale of the abscissa in (a) and (b) can be converted into moles (or equivalences) by dividing it by the molecular (or equivalent) weight of IgG (Table I). Likewise, the mass units in the scale of the abscissa the ordinate in (c) can be converted to moles (or equivalences) by the respective molecular (or equivalent) weight of lipid A and IgG.

Figure 2. The SRD data of new methylene blue (a) and ALF (b) with lipid A in agarose gel. Two precipitate rings were observed for new methylene blue, but only the data of the inner ring were presented. The scale of the abscissa can be converted into moles (or equivalences) by dividing it with the respective molecular (or equivalent) weight of new methylene blue and ALF.

Figure 3. The relation between the slope of the SRD data and the inverse of component B concentration in the agarose gel. Systems presented include new methylene blue-lipid A (a), ALF-lipid A (b) and new methylene blue-dextran sulfate, heparin, and SuBSA (c). The mass units involved in the scale of the abscissa and the ordinate can be converted into moles (or equivalences) by dividing it with the corresponding molecular (or equivalent) weight.

Figure 4. Effect of triethylamine (a) and that of temperature (b) on the SRD of new methylene blue with *E. coli* 026B6 LPS. The [LPS] in the agarose gel is 500 ug/ml in (a) and 50 ug/ml in (b).