HUMORAL AND CELLULAR ASPECTS OF THE NORMAL AND IMMUNE RESPONSE TO GLYCEROL TEICHOIC ACID IN THE RABBIT

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INTRODUCTION

Since the early work of Nuttall(1888) and Buchner (1889), there have been many studies demonstrating that the serum of normal animals contains antibody specific for a wide variety of bacteria. Very little work has been done, however, to study these antibodies at the cellular level. The main consideration of this investigation was to detect the lymphoid cells in the normal rabbit which produce antibody to glycerol teichoic acid. Since much work has been done with glycerol teichoic acid by our group and since natural antibodies to this antigen have been detected in most normal rabbits, as well as a number of other species(Chorpenning and Frederick, 1971), it was the antigen of choice. Another benefit of using glycerol teichoic acid has been that it was possible to sensitize erythrocytes without the use of conjugating agents.

One of the most significant advances in methods in immunology in recent years has been the single cell assay system of Jerne and Nordin(1963). Jerne's technique of localized hemolysis in gel has been used to detect antibody production <u>in vitro</u> to a variety of antigens. The localized hemolysis in gel technique is sensitive enough to study individual antibody-forming cells even in situat-

ions where only a few such cells are present among millions of non-antibody-forming cells. However, most investigations, if not all, have been primarily concerned with the cellular response following antigen administration. Modification of the Jerne plaque technique by Dresser and Wortis(1965) has extended its use, since now 7S, as well as 19S, antibodies can be detected at the cellular level. Therefore, this technique was employed to study antibody synthesis in lymphoid cells in normal animals.

As a further study, the cellular and humoral responses of the rabbit to teichoic acid injected intravenously were monitored at various times after immunization. Serum antibodies were characterized and comparisons made between those observed at the cellular and humoral levels. Comparisons were also made between the normal and the immunized animal.

LITERATURE REVIEW

Most definitions of antibodies assume that they are formed in response to an antigen and will react specifically with that antigen or with substances of similiar structure (Davis et al., 1968). Yet it has been known for many years that there are a vast array of serum proteins with the physicochemical and biological properties of immunoglobulins that react specifically with certain antigens even though the individuals in whom the globulins are found have had no known exposure to the antigens. These immunoglobulins are referred to as natural antibodies. Presently, the term natural antibody does not presuppose any particular origin, but much work is being done by our group in an effort to determine whether the formation of natural antibodies is under simple genetic control, thus being completely independent of immunogenic stimulation, or results from environmental stimuli of microbial, plant, and animal origin.

Since the early work of Nuttall (1888), Buchner (1889), and others first disclosed the bactericidal property of normal serum, a considerable amount of attention has been devoted to this important biological phenomenon. Studies in different species of animals have provided the bulk of

our early knowledge concerning the distribution and mode of action of natural antibacterial antibodies. Bordet (1899) reported the absorption of agglutinins specific for cholera vibrios and typhoid bacilli from normal horse serum. Burgi (1907) reported that agglutinins for Vibrio cholera, Salmonella typhi, Eschericha coli, Pasteurella aviseptica, Staphylococcus aureus, and Proteus vulgaris were present in the normal sera of several animals. Unfortunately, he did not demonstrate the specificity of his reported serological activity. Muir and Browning (1908) reported that the treatment of normal serum with increasing amounts of a dead emulsion of a bacterium usually results in the reduced bactericidal action on homologous reaction was reduced more, indicating some type of specific serum component. Systematic studies by Mackie and Watson (1926), Mackie and Finkelstein (1928, 1930), and Gibson (1930) demonstrated specific complement-fixing and agglutinating activity for a wide variety of bacterial antigens in the normal sera of various mammalian species. To demonstrate the high degree of specificity of these natural bactericidal antibodies, absorption tests were used. Gibson further reported that he found the serum of various young animals to be

deficient in agglutinating activity. Mackie and Finkelstein(1931, 1932) tested the serum of a number of normal animals against a variety of bacteria, including gram negative bacteria of typhoid-dysentary-vibrio groups and some gram positive organisms, and found both complement and bactericidal action in a large number of cases. They further showed that the bactericidal effect depends on the joint action of complement and a heat stable factor that could be removed by absorption. The specificity of the heat stable factor was demonstrated by removing the bactericidal activity against a particular organism by absorption and leaving the bactericidal activity for other bacteria. From these and previous observations, Mackie and Finkelstein suggested that normal serum contains naturally-occurring antibodies which are increased upon immunization. However, Gordon and Carter(1932), though finding that the bactericidal effect of rabbit and guinea pig sera depended on complement and a heat stable factor, were able to remove, nonspecifically, the bactericidal effect for a number of gram negative bacteria. They further demonstrated that variations in this activity of normal serum against different organisms depended upon the sensitivity of these bacteria to the nonspecific serum component.

Adler (1953) found bactericidins in the serum of normal guinea pigs and rabbits which aided in destroying S. typhosa. Upon further investigation, these bactericidins were found to be specific for the IX and XII surface antigens. Since typhoid antigens, IX and XII , are shared by many enteric bacteria, Adler suggested that subdetectable levels of antibody could be produced to these antigens, whatever their bacterial source. Thus the absorption of normal serum by a particular enteric strain may result in the removal of specific antibody with the consequent reduction in serum bactericidal power toward other enterics. This suggests that certain natural antibodies are able to react with a large number of bacteria.

In recent years, many highly sensitive tests have been used to demonstrate various natural antibodies and their specificities. For instance, Landy and Weidanz (1964) demonstrated constant levels of antibody against E. coli in specimens from rabbit and humans over a period of two years using bactericidal activity as a means for measuring antibody. Michael et al. (1962), using a standardized immune serum, reported that 0.0005 ug of antibody nitrogen can be detected by the bactericidal test. This test was also used by Landy and Weidanz (1964) to compare

the antibacterial activity against E. coli among eleven inbred strains of mice. As a result of this study, a wide variation of antibacterial activity was found among the different strains. Using gel diffusion, agglutination, and fluorescent antibody tests, Cohen(1963) was able to demonstrate natural antibodies to various strains of staphylococci in normal mice. Passive hemagglutination has been used extensively by Chorpenning et al.(1966, 1967, 1969, 1971) to demonstrate natural antibody to a bacillary antigen in a number of species. Therefore, with the advent of more highly sensitive tests, a greater number of natural antibodies with defined specificities are being demonstrated.

In some instances, the relative incidence of naturally-occurring antibodies in the serum of normal animals probably reflects the prevalence of the bacteria in the environment of the animals concerned. Upon investigating human gamma globulin, Millican et al.(1957) found it to be protective against S. aureus, Pseudomonas aeruginosa, E. coli, Proteus sp., and Klebsiella sp.. Fisher and Manning(1958) confirmed these observations, adding S. salivarius, S. pyogenes group A, and and S. pneumoniae to the list, but no protection could be demonstrated against S. typhi-murium, S. pyogenes group C, P. septica, or K. pneumoniae, four organisms that are not common inhabitants of the human body. Fisher (1959)

and Fisher and Manning (1961) demonstrated the specificity of the protective action by absorption. Neter et al. (1959) further investigated the widespread distribution of natural agglutinins to gram negative bacteria in pooled human gamma globulins harvested in different countries. They found a low incidence of antibody to Shigella dysenteriae and S. paratyphi A in preparations from the United States and Japan, where the corresponding infections are uncommon, and a high incidence in globulins from India, where the infections are prevalent. However, Abdoosh (1936) studied the bactericidal power of the serum against gonococcus in man and a large number of animals species. Anti-gonococcal antibody was low in normal human, mice, and guinea pigs, but high in horses, oxen, sheep, pigs, goats, cats, monkeys, rabbits, and rats (animals naturally insusceptible to the gonococcus). This brings up the question as to why animals naturally resistant to gonococcus have antibody, whereas man the common host of gonococcus, does not.

From the studies cited above, as well as others, there can be little doubt that normal animals produce specific antibodies for a wide variety of bacteria and that, in some instances, the antibody can be attributed to an external environmental stimulus.

However, even though an antibody results from an external antigenic stimulus, it may have been formed in response to antigens of an entirely different origin form those used to detect it. For example, the isoagglutinins associated with the different blood groups of man cross-react with bacterial antigens. Springer, Williamson, and Brandes (1961) showed that 50 percent of 282 aerobic gram negative bacteria, mainly inhabitants of the gut, inhibited A, B, or H(O) hemagglutination when added in suspension to the specific agglutinating system. In view of the absence in human blood of iso-antibodies for Rh_o(D), M, and N, it is significant that none of the bacteria inhibited hemagglutinins for these substances. Antigens resembling the A and B substances have also been found in horse saliva (Landsteiner and Chase, 1936), hog mucosa (Brahn et al., 1932), type XIV pneumococci (Neter and Sobotka, 1935), E. coli 086 (Osawa, 1959), and several other places in nature.

To obtain more specific information on the origin of antibacterial antibodies, and especially on the source and identity of the stimuli involved in their production, newborns and young animals were studied. Rywasch(1907) and Sherman(1919) noted that the normal hemagglutinins

of the fowl did not appear in the chick embryo until the twenty first day. Bailey(1923), in confirming the absence of hemagglutinins in the young chick and their appearance during growth, also found that chickens fail to respond to foreign red cells until they naturally develop some agglutinating capacity. Mackie and Finkelstein(1928) reported that the Wassermann-reacting properties of young rabbits were absent for 2-3 weeks, but appeared soon afterwards and that titers were progressive during development. Jordan(1937) reported the serum of calves to be devoid of agglutinating activity for Brucella and Salmonella, while adult cattle had such activity. Freund(1930) reported the failure of young rabbits to respond to bacteria, erythrocytes, or protein antigens. Therefore, the study of young animals does not tell us whether the gradual appearance of natural antibodies is due to increased exposure to external antigenic stimulus or to physiological ripening of the immune system as suggested by Hirszfeld (1926).

The development of germ-free conditions provided an opportunity to obtain information on the origin of natural antibodies in a situation where known external stimuli could be controlled. Wagner(1955) reported that germ-free chicks remained free of agglutinins against E. coli,

S. epidermis, and P. aeruginosa for one year, while conventional chicks developed antibodies. The absence of hemagglutinins for rabbit erythrocytes in germ-free chicks for up to 30 days of age, while these agglutinins were present in the sera of conventional chicks of the same age, was also reported by Wagner(1955). In both cases, no viable organisms could be demonstrated in the autoclaved diet. In view of these observations, Wagner (1959) investigated whether dead bacteria in the diet stimulated the production of agglutinins via the oral route. He found that dead Paracolobactrum aerogenoides incorporated into the diets of germ-free chickens stimulated agglutinin formation equal to that of conventional chickens raised on a diet without added dead organisms. Furthermore, killed P. aerogenoides in the diet of conventional chickens gave no significant agglutinin rise over the same diet containing dead organisms. Springer(1959) was able to demonstrate anti-B activity in germ-free chicks by feeding them a semi-synthetic diet containing killed E. coli O_{86}. While germ-free chicks fed a semi-synthetic diet free of demonstrable blood group activity showed no agglutinins up to 60 days of age. Even by 90 days of age, agglutinin titers in germ-free chickens amounted to only 10 percent of that found in ordinary

chickens of the same age. Having studied colostrum-deprived piglets under sterile conditions, Sterzl (1962), using complement fixation, opsonification, and passive hemolysis, reported no bactericidal action against E. coli and Salmonella prior to immunization. However, Landy et al. (1962) reported no differences in the levels of bactericidal antibodies against S. typhosa between germ-free and conventional mice. In a later study, in which the test animals were raised on a diet of autoclaved cow's milk rather than the conventional diet, Landy and Weidanz (1964) reported lower antibody levels against S. typhosa in germ-free mice than in conventional mice. Ikari (1964) also observed less antibody in germ-free than conventional mice.

The occurrence of natural antibacillary antibodies in almost all normal human sera was first reported by Chorpenning and Dodd (1965, 1967), using passive hemagglutination tests. Rozmiarek et al. (1969) found antibacillary antibodies were present also in both conventional and in germ-free guinea pigs raised on an autoclaved diet, supplemented with Vitamin C. In fact, the results indicated a higher titer in the germ-free pigs than in the conventional pigs. Upon further investigation, Frederick (1969) demonstrated two kinds of killed gram positive bacteria in Rozmiarek's autoclaved diet. It is thus

apparent that one must first remove all antigenic stimuli for the antibody in question before studies of the occurrence of natural antibodies in germ-free animals are likely to answer the question of whether or not natural antibodies can arise spontaneously.

Recently, an attempt has been made to localize and study the cells producing natural antibody. Using localized hemolysis in gel, Sterzl et al. (1965) reported that by the fourteenth day after birth up to 277 cells per spleen were observed to be producing antibodies to sheep red cells in piglets raised under normal conditions. In contrast under germ-free conditions at one month of age, Sterzl was not able to demonstrate antibody producing cells. In studying the mouse, Wigzell (1966) found that the majority of naturally-occurring hemolytic-plaque-forming cells against sheep erythrocytes were found in the spleen rather than other lymphoid patches, and that only 19S antibody was demonstrable. Hege and Cole (1967) were able to show that after whole body x-irradiation, at levels known to inhibit cellular proliferation, and after neonatal thymectomy the number of cells producing natural antibody against sheep and horse red blood cells was not decreased.

Since the present investigation concerns naturally-occurring antibodies to teichoic acid, observations regarding this antigen will now be reviewed. The observations of Mitchell and Moyle (1951) that cell envelopes of S. aureus contain unusually large amounts of phosphorus, led eventually to the discovery of teichoic acids. Subsequently, Baddiley and his co-workers (1958) isolated teichoic acids of differing chemical composition from a number of gram positive bacteria. Two basic types of teichoic acid have been found by Baddiley's group: polyglycerophosphate linked 1,3 by phosphate ester, and polyribitolphosphate linked 1,5 by phosphate esters. Rantz et al. (1952, 1956) first reported that culture filtrates and acid or phenol extracts from streptococcal Groups A, C, G, and D, as well as from S. viridans, S. aureus, Bacillus sp., and some pneumococci contained a cross-reacting substance. They further reported that the substance was generally absent from gram negative bacteria. In subsequent work, Neter (1959, 1960) demonstrated that red cells modified by extracts of S. aureus, B. subtilis, and Listeria monocytogenes would react with staphylococcal antisera, further demonstrating the cross-reactivity of their antigen. After isolating and purify-

ing a substance from S. pyogenes similiar to the Rantz antigen, McCarty (1959) first suggested that the antigenic component may be polyglycerophosphate. Chorpenning and Dodd (1966) were able to modify human erythrocytes in vitro with material extracted from a Bacillus sp. (designated Bacillus sp. 372-56) and to demonstrate cross-reactions with staphylococci and streptococci, suggesting that it was similiar to the Rantz antigen. Later, Anderson et al. (1967) showed that the agglutination of erythrocytes sensitized with the extract of Bacillus sp. 372-56 could be inhibited with synthetic polyglycerophosphate. This observation suggested that one of the active substances in the extract was polyglycerophosphate. Schmidt (1968) further purified the Bacillus sp. 372-56 extract while maintaining its capability of modifying erythrocytes. After chromatographic analysis of the hydrolysate, he suggested that the major constituent of the extract was a polymer of glycerophosphate. However, some protein remained in the final product as indicated by the Folin-Ciocalteu test and ultraviolet absorption-spectrum analysis. Decker et al. (1971) confirmed this suggestion and found the antigen to be specific for glycerol teichoic acid in passive hemagglutination tests.

Only a few studies have been made of natural antibodies to chemically defined and purified teichoic acids. Using microquantitative precipitation techniques, Torii et al.(1964) demonstrated anti-ribitol teichoic acid antibodies in four individuals with no previous history of staphylococcal infection. Daugherty et al.(1967) demonstrated by gel diffusion that anti-ribitol teichoic acid antibodies were frequently found in concentrated normal human sera and in unconcentrated sera of patients with staphylococcal disease of more than 30 days duration. Using passive hemagglutination, Chorpenning et al.(1969) demonstrated that a high frequency of normal human, rabbit, sheep, swine, and guinea pig sera contain antibodies reacting with a partially-purified extract of Bacillus sp. 372-56 which appeared to be glycerol teichoic acid. Later, Chorpenning and Frederick(1971) confirmed this work with a purified extract and presented preliminary data on the characteristics of the natural antibodies in several species. It is obvious more work needs to be done with teichoic acids of defined specificities, especially at the cellular level.

PART I PRELIMINARY EXPERIMENTS

MATERIALS AND METHODS

Experimental Plan

Although the Jerne technique(1963) of localized hemolysis in gel is an excellent method for recognition, enumeration, and other studies of individual antibody forming cells, a modification utilizing antigen-coated red blood cells must be employed if antibodies to soluble antigens are sought. Furthermore, in studies of natural antibodies, very low concentrations of plaque-forming cells may be expected and high sensitivity is a necessity. This latter requirement is complicated by the desirability of using erythrocytes of the same species in such tests. Therefore, in order to obtain maximum plaque formation in these studies of natural antibodies to teichoic acid, several preliminary experiments were conducted. The first was a study of red cell modification with varying amounts of antigen to determine the optimum concentration to be used in passive hemolysis. Next, was a study comparing the relative hemolytic sensitivity of modified sheep erythrocytes, using guinea pig serum as a complement source, with modified rabbit erythrocytes, using complement from various species. Finally, various types of buffers and agars were compared for

maximum plaque formation under a standard set of conditions.

Another limitation was that only lymphoid cells producing IgM are capable of efficiently lysing erythrocytes. Other classes of antibody, IgG and IgA, are capable of agglutinating erythrocytes but cause very little hemolysis. Dresser and Wortis(1965) have shown that these other classes of antibody form plaques if an anti-globulin developing serum is used. The addition of anti-IgG, for example, will result in plaques specific for both IgG and IgM. Therefore, to determine the number of plaques specific for IgG, the number of plaques on plates without anti-globulin was substracted from the number of plaques on plates with the antiserum.

Animals

Normal adult albino rabbits weighing over 2.0 kg were used for these studies.

Antigen

The antigen preparation used for erythrocyte modification was derived from Bacillus sp. 372-56 (Chorpenning and Dodd, 1966) and was made available by David H. Schmidt. This preparation was shown by Decker and Chorpenning

(1971) to be specific for glycerol teichoic acid in passive hemagglutination tests.

Passive Hemolysis

The principle of the passive hemolysis reaction is that hemolysis occurs when antibody reacts in the presence of complement with a soluble antigen fixed to the surface of erythrocytes. As such, it is an excellent tool for estimating the optimal conditions for localized hemolysis to occur in gel.

Previous to modification, whole sheep blood in Alsever's solution or fresh whole rabbit blood was washed three times in 20 volumes of phosphate-buffered saline(0.85% NaCl,buffered with M/15 Disodium and Monopotassium phosphates) at pH 7.3. Modification was accomplished by adding 0.1 ml of the washed and packed red cells to 1.9 ml of phosphate-buffered saline (PBS) containing 500 ug/ml of antigen. The erythrocyte suspension was adjusted to a concentration of 5% as determined spectrophotometrically after adding 0.1 ml of the suspension to enough distilled water to bring the final volume to 2 ml. The resulting lysates were read against a distilled-water blank at a wavelenght of 530 mu in a Bausch and Lomb Spectronic 20 spectrophotometer. An optical density of 0.45 $\pm$ 0.005 corresponded to a 5% suspension of red cells. The antigen-containing suspen-

sion was then incubated at 37°C for 30 minutes with frequent agitation. The red cells were centrifuged at 1000 rpm, washed thrice in 5 volumes of cold PBS, and resuspended in cold PBS (pH 7.3) to the desired concentration.

The protocol for setting up a passive hemolysis assay is presented in Table 1. Titer (PH_{100}) of the antiserum was determined as the reciprocal of the highest dilution causing 100% lysis of the red cells.

Table 1.

Protocol of the Passive Hemolytic Test

Reagents	Row I	Row II	Controls I	Controls II
antiserum diluted serially in PBS at pH 7.3	0.05	0.05	-	-
PBS at pH 7.3	-	-	0.05	0.05
1% modified erythrocytes	0.10	-	0.10	-
1% unmodified erythrocytes	-	0.10	-	0.10
incubate at 37°C for 30 minutes				
complement, $C'H_{50}$ unit	0.05	0.05	0.05	0.05
incubate at 37°C for 30 minutes				

Complement

Lyophilized guinea pig serum from Grand Island Biologicals was reconstituted with 0.15 M NaCl and stored at -20°C to minimize the loss of complement activity.

For this study, complement activity was assayed by a modification of the technique of Bier et al.(1952) in terms of 50% hemolytic units ($C'H_{50}$) or the amount of complement required to lyse approximately 50% of the red cells under standard conditions. For this determination, serial dilutions of complement were made in triethanolamine-buffered salt solution (TBS) and 1 ml of each was placed in separate tubes. As controls for hemolysis, 1 ml of undiluted complement was added to each of three tubes. A blank containing 1 ml of TBS was also included. Then 3.2 ml of a standard anti-teichoic acid antiserum was added to 3.2 ml of a standardized 1% modified erythrocyte suspension. The contents were mixed thoroughly and were incubated ten minutes at room temperature. To each tube containing complement, as well as the blank, 0.50 ml of the sensitized cells were added, mixed well, and incubated for 30 minutes at 37°C. After the incubation period, 0.50 ml of chilled TBS was added to each tube which was then centrifuged at 1000 X g for 5 minutes. Optical densities for each tube was determined in calibrated 12 x 75 mm

cuvettes, and the results read spectrophotometrically at 550 mu against the TBS blank. The degree(%) of hemolysis for each complement dilution was then plotted on a graph. The $C'H_{50}$ hemolytic unit was determined by reading the corresponding volumes of complement-containing serum from the curve. The accuracy of the titration was confirmed by pipetting 0.06 ml volumes of a $C'H_{50}$ unit into three 12 x 75 mm cuvettes and adding 0.84 ml of cold TBS and 0.60 ml of sensitized cells to each. The contents of the tubes were mixed and incubated in a water bath for 30 minutes at 37°C. Following incubation, 0.50 ml of cold TBS was added, the contents mixed, and the tubes centrifuged to sediment the unlysed cells. Using the controls of the initial complement titration, the optical densities of the supernates were determined spectrophotometrically and the percentage of hemolysis calculated for each. Tests indicated that each tube yielded between 45-55 % hemolysis.

Passive Hemagglutination

Hemagglutination tests were performed by serially diluting the rabbit serum in phosphate-buffered saline (pH 7.3) and mixing 0.05 ml of each dilution with 0.05 ml of a 1% suspension of modified rabbit erythrocytes in phosphate-buffered saline at pH 7.3. After incubating the

mixture for 30 minutes at 4°C, each tube was centrifuged for 15 seconds in an Adams Serofuge and the amount of agglutination determined using a dissecting microscope (20X). The following criterion was used for detecting the degree of agglutination:

4 ≠ - Cells in one clump.

3 ≠ - Cells in large clump with several other smaller clumps.

2 ≠ - Many small clumps.

1 ≠ - Very little clumping with many single cells.

\- - All single cells, no clumping.

As a means of sensitivity control, all tests included a standardized pre-diluted immune rabbit serum. Each undiluted sera was also tested with a 1% suspension of unmodified rabbit erythrocytes.

Lymphoid Cell Suspensions

Rabbit spleens and several mesenteric lymph nodes were delivered into Hank's balanced salt solution (HBSS) on ice and were promptly minced with sterile scissors. The minced whole organs were homogenized in a loose-fitting glass homogenizer and the resulting mixture was filtered through four layers of surgical gauze into a

test tube. The mesenteric lymph node cells were washed three times in 5 volumes of cold HBSS by centrifugation at 1000 rpm for 3 minutes. Spleen cells were washed once in HBSS and resuspended in 0.83% aqueous ammonium chloride solution. After standing 10 minutes at room temperature, the cells were washed 3 times in 5 volumes of cold HBSS at 1000 rpm. This procedure removed rabbit erythrocytes, which interfere with plaque production (Daniels and Weigle, 1968). The suspension were held on ice until assayed.

Cells processed in this manner were counted in a hemocytometer and were shown to be 85 to 90% viable, as judged by their ability to exclude 0.5% trypan blue.

Antisera

Goat anti-rabbit IgG and goat anti-rabbit IgA were obtained from Miles Laboratories, Inc., Illinois. According to Research Products Catalog C, all antisera had been standardized by immunoelectrophoresis, immunodiffusion, and protein assays.

Localized Hemolysis in Gel

Two layers of gel were used. The bottom layer was to provide a smooth non-toxic level surface for the top layer which contained the lymphoid cells and the indicator red blood cells. This bottom layer consisted of 10 ml

of 1.2% ion agar in Dulbecco's phosphate-buffered saline (PBS) and 0.05 g/ml DEAE dextran. The agar was then liquefied by autoclaving at 20 lbs pressure for 10 minutes and poured into a 9 cm disposable polystyrene Petri dishes. These prepared plates were either used at once or stored up to 48 hours at $4^{o}C$. For the top layer, 2 ml of 0.6% agarose in Gey's solution were pipetted into sterile test tubes and maintained in a water bath at $47^{o}C$. After the cells were prepared, a tube was removed from the bath and 0.1 ml of the 20% modified sheep red blood cell suspension was pipetted into it. The contents were mixed and 0.1 ml (1×10^{6} cells) of the lymphoid cell suspension was added. The contents were mixed again and poured into the prepared Petri dish. After the top layer was spread by a swirling motion and allowed to set on a level surface, plates were incubated for 1 hour at $37^{o}C$ in a moist atmosphere of 5% carbon dioxide and 95% air. After this period, 2 ml of a 1:100 dilution of a specific goat anti-rabbit IgG or goat anti-rabbit IgA serum was pipetted on to the surface of the plate and gently spread. The plates were incubated for 1 hour at $37^{o}C$. The added goat serum was then poured off and 2 ml of a stated dilution of guinea pig serum was added to each plate as a source of comple-

ment. The plates were incubated for 30 minutes at 37°C and the complement was then poured off. The plaques were counted after storage for no longer than 24 hours at 4°C. Duplicate assays were performed for determination of background activity to unmodified sheep red blood cells. Normal rabbit serum at the same dilution as that of the developing antiserum was added as a control. Addition control experiments were performed without the addition of either antiserum of normal serum.

Effect of Enhancing Sera

A dilution of 1/100 for each anti-heavy chain antiserum was used in detecting indirect plaques. To demonstrate the immunologic specificity of enhancement with each antiserum, six plates were prepared; three plates contained modified sheep cells in the top layer and three plates contained unmodified sheep cells. Antisera from a rabbit immunized with glycerol teichoic acid was fractionated and each fraction examined by immunoelectrophoresis against specific antisera. Purified IgM and IgG fractions, demonstrating passive hemagglutinating activity for glycerol teichoic acid were then adsorbed with sheep red blood cells. To each of six plates, 0.05 ml

of each fraction was added and incubated at 37°C for 1 hour. Two plates, one containing unmodified sheep cells and one containing modified sheep cells, were then treated with complement and incubated. As a means of comparison, two plates were treated with goat anti-rabbit IgG and two plates with goat anti-rabbit IgA. After 1 hour incubation at 37°C, these plates were also treated with guinea pig serum.

RESULTS

Optimal Antigen Concentration for Erythrocyte Modification

Various concentrations of antigen were tested for activity by checkerboard titration and the modifying activities were compared using passive hemagglutination (Table 2) and passive hemolysis (Table 3).

Table 2

Determination of the Optimal Antigen Concentration for Erythrocyte Modification Using Passive Hemagglutination

Antigen in	Dilution of Standard Antiserum						
g/ml	1/1	1/2	1/4	1/8	1/16	1/32	1/64
4.00×10^{-3}	4 ≠	4 ≠	4 ≠	3 ≠	3 ≠	3 ≠	1 ≠
2.00×10^{-3}	4 ≠	4 ≠	4 ≠	4 ≠	4 ≠	3 ≠	1 ≠
1.00×10^{-3}	4 ≠	4 ≠	4 ≠	4 ≠	4 ≠	3 ≠	1 ≠
5.00×10^{-4}	4 ≠	4 ≠	4 ≠	4 ≠	3 ≠	3 ≠	1 ≠
2.50×10^{-4}	4 ≠	4 ≠	4 ≠	4 ≠	3 ≠	3 ≠	1 ≠
1.25×10^{-4}	4 ≠	4 ≠	4 ≠	4 ≠	4 ≠	4 ≠	1 ≠
6.25×10^{-5}	3 ≠	3 ≠	1 ≠	1 ≠	1 ≠	-	-
3.28×10^{-5}	3 ≠	3 ≠	1 ≠	-	-	-	-
1.69×10^{-5}	2 ≠	2 ≠	-	-	-	-	-

Table 3

Determination of the Optimal Antigen Concentration for Erythrocyte Modification Using Passive Hemolysis

Antigen in g/ml	Dilution of Standard Antiserum 1/1	1/2	1/4	1/8	1/16	1/32	1/64
4.00×10^{-3}	≠	≠	≠	≠	-	-	-
2.00×10^{-3}	≠	≠	≠	≠	≠	-	-
1.00×10^{-3}	≠	≠	≠	≠	≠	-	-
5.00×10^{-4}	≠	≠	≠	≠	≠	-	-
2.50×10^{-4}	≠	≠	≠	≠	≠	-	-
1.25×10^{-4}	≠	≠	≠	≠	≠	-	-
6.25×10^{-5}	≠	≠	≠	≠	-	-	-
3.28×10^{-5}	≠	≠	≠	≠	-	-	-
1.69×10^{-5}	≠	≠	≠	≠	-	-	-

Using passive hemagglutination, a plot of the endpoint titers versus antigen concentrations shows that modifying activity did not increase with an increase in antigen concentration beyond 125 ug/ml of phosphate-buffered saline at pH 7.3(Figure 1). Whereas using passive hemolysis, modifying activity changed very little with any of the concentrations of antigen used(Figure 2). In the present studies, red cells for all tests were treated with 500

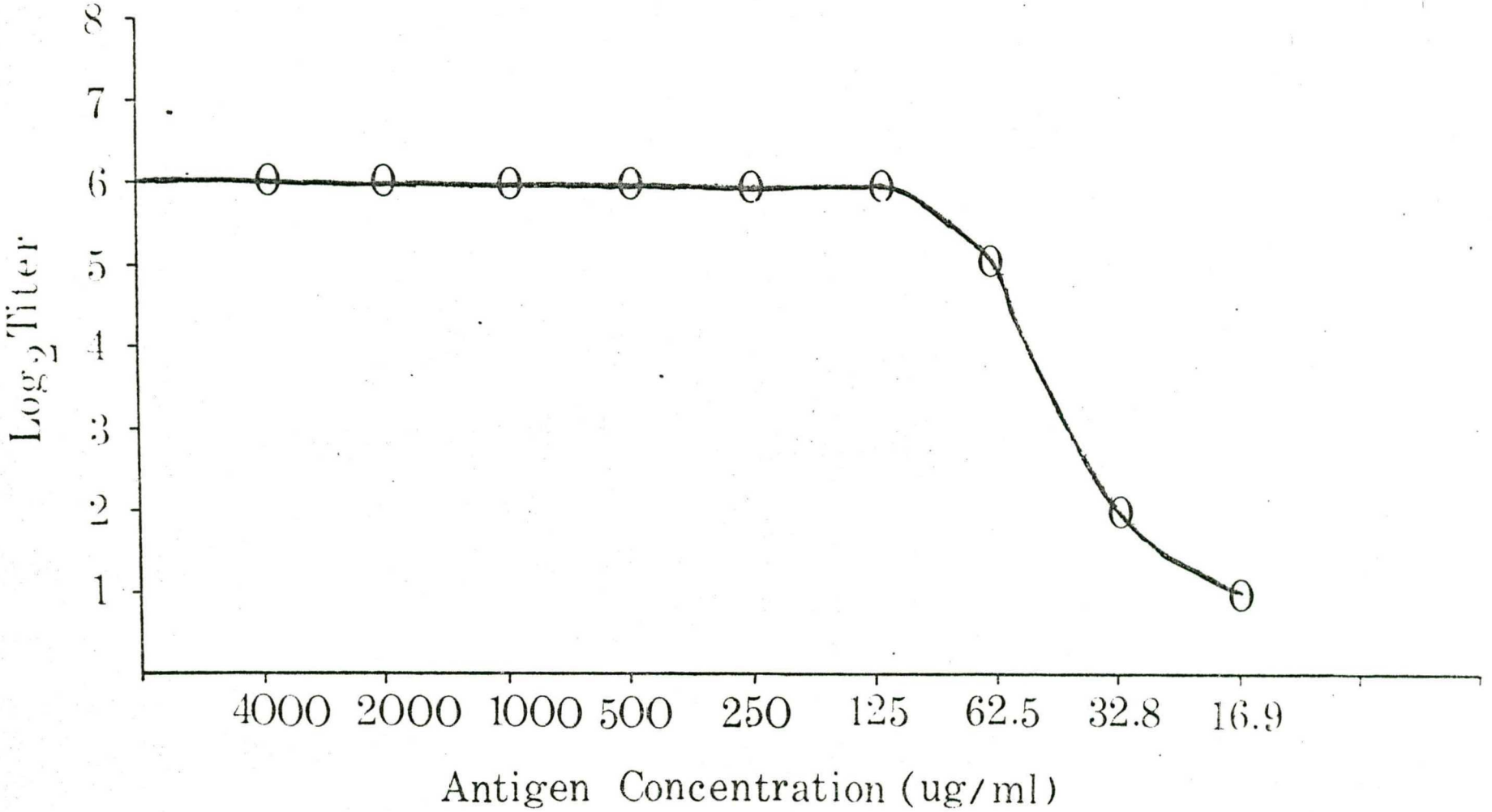

Figure 1 Determination of the Optimal Antigen Concentration for Erythrocyte Modification by Passive Hemagglutination

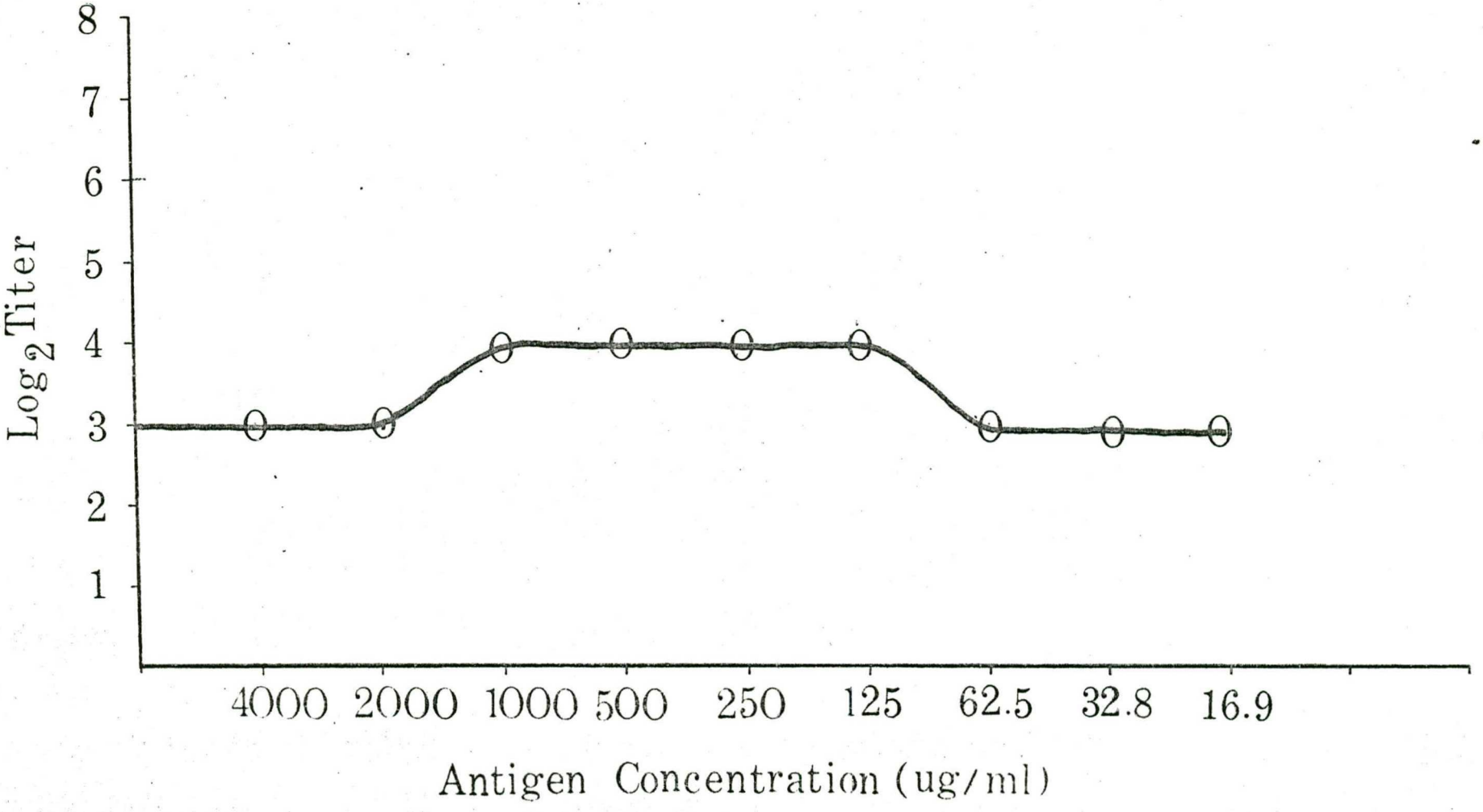

Figure 2 Determination of the Optimal Antigen Concentration for Erythrocyte Modification Using Passive Hemolysis

ug of antigen/ml for convenience and to insure ample modifying activity.

Complement Titration

The $C'H_{50}$ unit was chosen as a means of titrating complement. Rather than using sheep blood cells and an anti-sheep hemolysin system, modified sheep red blood cells were used and a standard rabbit antiserum containing anti-teichoic acid antibodies. To remove anti-sheep activity, the standard serum was adsorbed with sheep erythrocytes. Table 4 contains the results of a standard guinea pig complement used throughout this work.

Table 4

Results of Guinea Pig Serum Titration

	Dilutions of Standard Antiserum							
	1/2	1/4	1/8	1/16	1/32	1/1	1/1	1/1
optical densities	0.40	0.25	0.19	0.17	0.15	0.66	0.68	0.64
% hemolysis	61	38	29	26	23	100	100	100

Figure 3 is a plot of the volume of complement per ml of 0.15 M NaCl versus the corresponding percentages of hemolysis. After fitting a line to the experimental points,

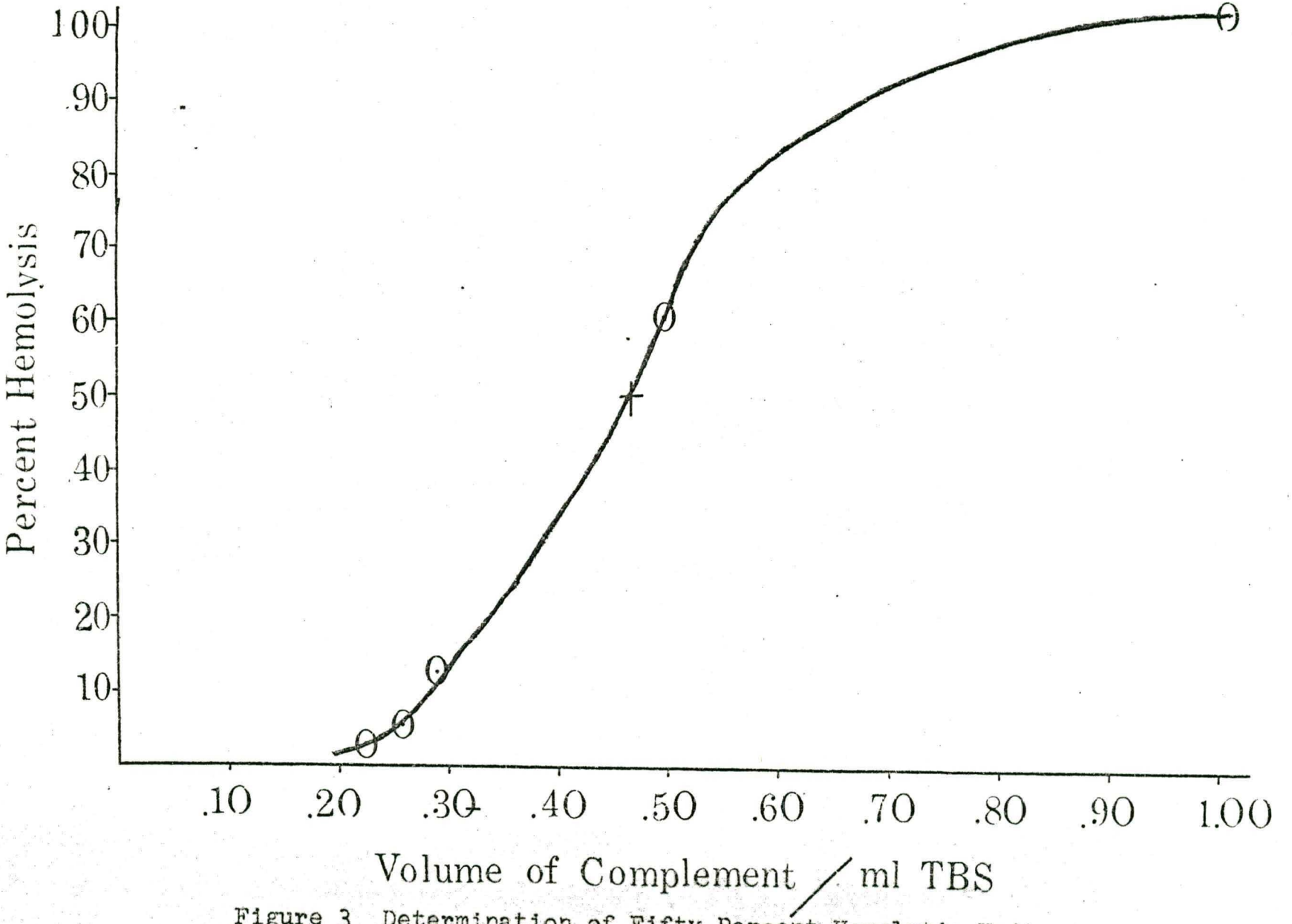

Figure 3 Determination of Fifty Percent Hemolytic Unit

it can be seen that 0.47 ml of complement(Lot 08114 W) per total volume of 1 ml provides a $C'H_{50}$ unit.

Homologous Erythrocytes Versus Heterologous Erythrocytes

Using passive hemolysis, a series of experiments were performed with homologous coated erythrocytes and various types of complement since it was desired to use such cells in order to cancel out the normal anti-sheep hemolysins. The $C'H_{50}$ units determined for 1 ml of human, guinea pig, rat, and mouse sera, using modified sheep cells and a standard rabbit antiserum, are shown in Table 5. Results of passive hemolysis tests using modified rabbit cells with various complement sources are also presented. They indicate that a system employing modified sheep red blood cells and guinea pig complement was more efficient in producing hemolysis than modified rabbit red cells with any other types of complement. It should be noted that the immune standard rabbit serum used for these determinations was of higher titer, as determined by passive hemagglutination, than the standard immune rabbit serum used in titrating the various types of complement. As a result of this work, sheep erythrocytes were employed as the indicator cell and guinea pig serum as a source of complement in subsequent work.

Table 5

Comparisons of Indicator Cells and Complement Sources

Complement Source	$C'H_{50}$ Units*	Indicator Cell	PH_{100} Titer
guinea pig serum	2.7	sheep RBC	8
guinea pig serum	2.7	rabbit RBC	1
rat serum	1.8	rabbit RBC	0
mouse serum	1.5	rabbit RBC	0
human serum	2.0	rabbit RBC	2

* modified sheep cells as the indicator system

Composition of Gel and Buffer

The relative number of plaque-forming cells specific for glycerol teichoic acid from a normal rabbit lymphoid cell suspension depends on the buffers used in the assay plates and the composition of the gel. Comparing the various buffers and gels listed in Table 6, it was apparent that a top layer of agarose mixed in Gey's solution and a bottom layer of ion agar mixed in Dulbecco's phosphate-buffered saline and 0.5 mg/ml DEAE dextran provided the best results. Demonstration of plaque-forming cells producing IgM antibodies to glycerol teichoic acid could

Table 6

Effect of Composition of Gel or Buffer on Number of Observed Plaques

Top Layer		Bottom Layer		No. of	Direct PFC/Plate
Gel	Buffer	Gel	Buffer	Animals	Mean**
Difco Agar	Phosphate Buffered Saline pH 7.3	Difco Agar	Phosphate Buffered Saline pH 7.3	2	0
Difco Agar	Earle's BSS*(199)	Difco Agar	Earle's BSS (199)	2	0
Difco Agar	Earle's BSS (199) ≠ 0.5 mg/ml DEAE	Difco Agar	Earle's BSS (199) ≠ 0.5 mg/ml DEAE	2	0
Ion Agar	Hank's BSS	Ion Agar	Hank's BSS	2	0
Agarose	Hank's BSS	Agarose	Hank's BSS	4	3
Agarose	Eisen's Media	Agarose	Eisen's Media	2	0
Agarose	Gey's Solution	Ion Agar	Dulbecco's Phosphate Buffered Saline pH 7.3 ≠ 0.5 mg/ml DEAE	3	12

* Balanced Salt Solution

** Mean = $\text{antilog} \frac{\log (x \neq 1)}{n} - 1$

also be observed using a top and bottom layer of agarose mixed in Hank's balanced salt solution but the test was less sensitive.

Indirect Plaques

Plaques due to hemolytically inefficient rabbit IgG and IgA were developed using goat anti-rabbit IgG and IgA to enhance complement fixation and hemolysis. Figure 4 (a-f) presents experiments showing that the specificity of localized hemolysis in gel was specific for teichoic acid. In the first set of plates (a and b), hemolysis occurred only where rabbit IgM of anti-teichoic acid specificity adsorbed with sheep red blood cells has been deposited and only on the plate containing modified sheep erythrocytes. However, in the second series of plates (c and d), after the addition of anti-rabbit IgG, hemolysis can be seen in the areas where both IgM and IgG anti-teichoic acid antibodies were deposited. Again hemolysis occurred only on the plates containing modified sheep red blood cells. On the last set of plates (e and f), enhanced with goat anti-rabbit IgA, hemolysis was observed only on the area spotted with rabbit IgM containing antibodies for teichoic acid and only on the plates containing modified sheep cells.

Figure 4 Observable Effects of the Enhancing Sera

a) Plate containing modified sheep erythrocytes treated with guinea pig complement

   1 - Rabbit 19S anti-teichoic acid antibody adsorbed with sheep erythrocytes

   2 - Rabbit 7S anti-teichoic acid antibody adsorbed with sheep erythrocytes

b) Plate containing unmodified sheep erythrocytes treated with guinea pig complement

c) Plate containing modified sheep erythrocytes treated with guinea pig complement and goat anti-rabbit IgG

d) Plate containing unmodified sheep erythrocytes treated with guinea pig complement and goat anti-rabbit IgG

e) Plate containing modified sheep erythrocytes treated with guinea pig complement and goat anti-rabbit IgA

f) Plate containing unmodified sheep erythrocytes treated with guinea pig complement and goat anti-rabbit IgA

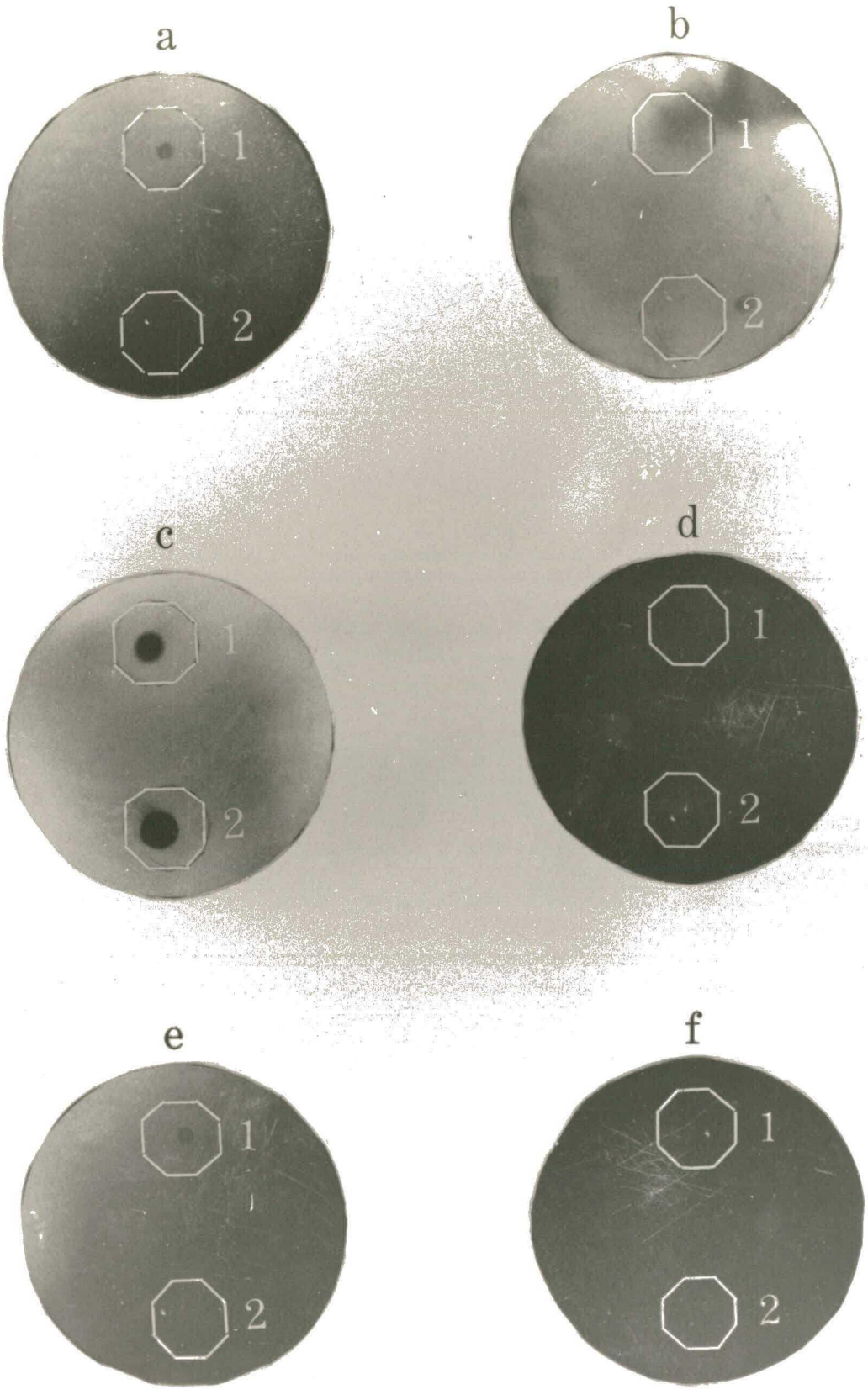

Figure 4 - Observable Effects of the Enchancing Sera

DISCUSSION

The ease with which sheep erythrocytes can be coated with glycerol teichoic acid and the efficiency of the coated cells in immune hemolysis were indications for the applicability of the technique of localized hemolysis in gel to the investigation of antibodies to teichoic acid at the cellular level.

Attempts to use rabbit erythrocytes as the indicator cell in the plaque assay to eliminate background activity resulting from natural antibodies directed against heterologous red cells were not successful. This agrees somewhat with Landy et al.(1965) who found that rabbit red blood cells could participate in immune hemolysis with guinea pig complement, but were not as sensitive to lysis as sheep erythrocytes. These were tube tests and no other types of complement were used by Landy's group. It therefore appears that susceptibility to hemolysis differs between rabbit and sheep erythrocytes.

Several alternative methods exist for carrying out localized hemolysis in gel. For tests with modified erythrocytes, a top layer of agarose in Gey's solution and a bottom layer of ion agar in Dulbecco's phosphate-buffered saline provided the greatest sensitivity. This may have been due to the greater efficiency of the complement in

in this type of medium. It has been known for some time that calcium and magnesium ions influence the efficiency of hemolysis by complement(Mayer, 1946). Daniels and Weigle(1968) further suggested that the concentration of calcium and magnesium ions in the media influence the efficiency of complement in localized hemolysis in gel.

The specificity of the plaques developed using goat anti-rabbit IgG and IgA was demonstrated(Figure 4). In addition, the specificity of modified sheep red blood cells was also demonstrated since no lysis was observed with unmodified sheep erythrocytes. Sell et al.(1970) found that the amplifying antiserum will, in some cases, inhibit the detection of direct plaque-forming cells. However, the enhancing sera did not appear to have any inhibitory effects on the direct plaques using our system.

PART II THE CELLULAR AND HUMORAL RESPONSES

MATERIALS AND METHODS

Animals

Both male and female adult albino rabbits weighing over 3 kg were used for these studies. The rabbits were housed one to a cage and fed pelletted rabbit chow.

Immunization and Bleeding

A suspension of Bacillus sp. 372-56 containing 4×10^9 cells/ml of phosphate-buffered saline pH 7.3 was prepared and autoclaved for 10 minutes. The animals were immunized by injecting 2 ml of the Bacillus sp. 372-56 suspension via the marginal ear vein. At various times after immunization, the animals were bled by cardiac puncture and sacrificed by exsanguination. After the whole blood was refrigerated for 24 hours, the serum was drawn off and clarified by centrifugation. The serum was then stored at -20°C without preservatives until use. The spleens and mesenteric lymph nodes were harvested immediately after exsanguination.

Characterization of Normal and Immune Sera

Fractionation of each serum sample was accomplished by using the gel filtration technique. After swelling Sephadex G-200 (Pharmacia Fine Chemicals, New Jersey),

exclusion limit about 200,000 , for 3 days in phosphate-buffered saline pH 8 and 0.02 NaN_3 as a preservative, the slurry of swollen G-200 was added to a 2.5 x 45 cm. column until filled. After the buffer was allowed to flow overnight to permit the gel beads to settle, a sample applicator was placed on the gel surface. The void volume was determined using a 0.5 % sample of Blue-Dextran 2000, molecular weight 2,000,000. The protein distribution of the effluent was determined by quantitative ultraviolet absorption measurements with an LKB Ultraviolet Absorptiometer set at 254 mu. This information was graphically recorded on a LKB type 6520H recorder and the effluent collected in 2 ml. portions in a refrigerated fraction collector. When whole serum was treated in this manner, three major peaks of protein were found in the effluent and could be collected by correlating volume markers on the graphic readout with the tubes collected.

Since the proteins were eluted from the column in relatively large volumes of buffer, each fraction was concentrated by ultrafiltration using a Model 50 DIAFLO apparatus with a XM-50 membrane filter (Amicon Corporation, Massachusetts). Each fraction was washed three times in

10 ml of phosphate-buffered saline at pH 7.3 and resuspended to the original volume of the whole serum when applied to the column.

Immediately after separation, immunoelectrophoresis was used to determine the relative purity and further characterize each fraction. Microscope slides were coated with a 1% solution of Noble agar in barbital buffer pH 8.2. The pattern outlined in the LKB Operating Manual(1965) was used in preparing the slides for immunoelectrophoresis. An LKB immunoelectrophoresis apparatus (LKB Produkter, Sweden) was set at 250 volts for 1 hour after the addition of the antigen to the wells. At the termination of the electrophoretic separation, 0.05 ml of specific antiserum was added to each trough. The antisera used were goat anti-rabbit IgM, IgG, and IgA obtained from Research Products Division. Each antiserum was standardized by immunoelectrophoresis, immunodiffision, and protein assay by the supplier.

To further characterize the antibodies, portions of each fraction and of the whole serum were treated with an equal volume of 0.2 M 2-mercaptoethanol for 30 minutes at 37°C. Each treated sample was then concentrated to its original volume in a DIAFLO cell using a PM-10 membrane filter.

To estimate the stability of anti-teichoic acid antibody in a particular fraction, treated samples were tested in parallel with untreated samples by passive hemagglutination. Since 2-mercaptoethanol treatment selectively inactivates 19S antibody specific for teichoic acid would demonstrate a reduction in passive hemagglutination titer as compared to untreated samples.

RESULTS

The Normal and Immune Humoral Response in the Rabbit

Anti-glycerol teichoic acid antibodies appeared regularly in the serum of unimmunized rabbits. As shown in Figures 5 and 6, these antibodies were found only in the 19S fraction from Sephadex separation of the serum. A single intravenous injection of Bacillus sp. 372-56 did not regularly produce an increase in humoral antibody during the first 15 days, although some titers were higher than the average normal titer (Figures 5-12). By the 15th day, antibody appeared in Sephadex Fraction II (7S) and was shown to be resistant to treatment with 2-mercaptoethanol, while earlier antibody in Fraction I (19S) was susceptable to such treatment (Table 7). Figures 13-17 are representative immunoelectrophoretic patterns of the fractionated sera. After examining these patterns, it appears that Fraction I was essentially IgM and Fraction II was essentially IgG. In every serum sample the only demonstrable immunoglobulin in Fraction I was IgM and the only one in Fraction II was IgG. However, Fraction III, in almost every case, contained IgG antibody. It should also be noted that the precipitation lines for IgM antibody were very faint and that IgA antibody was not demon-

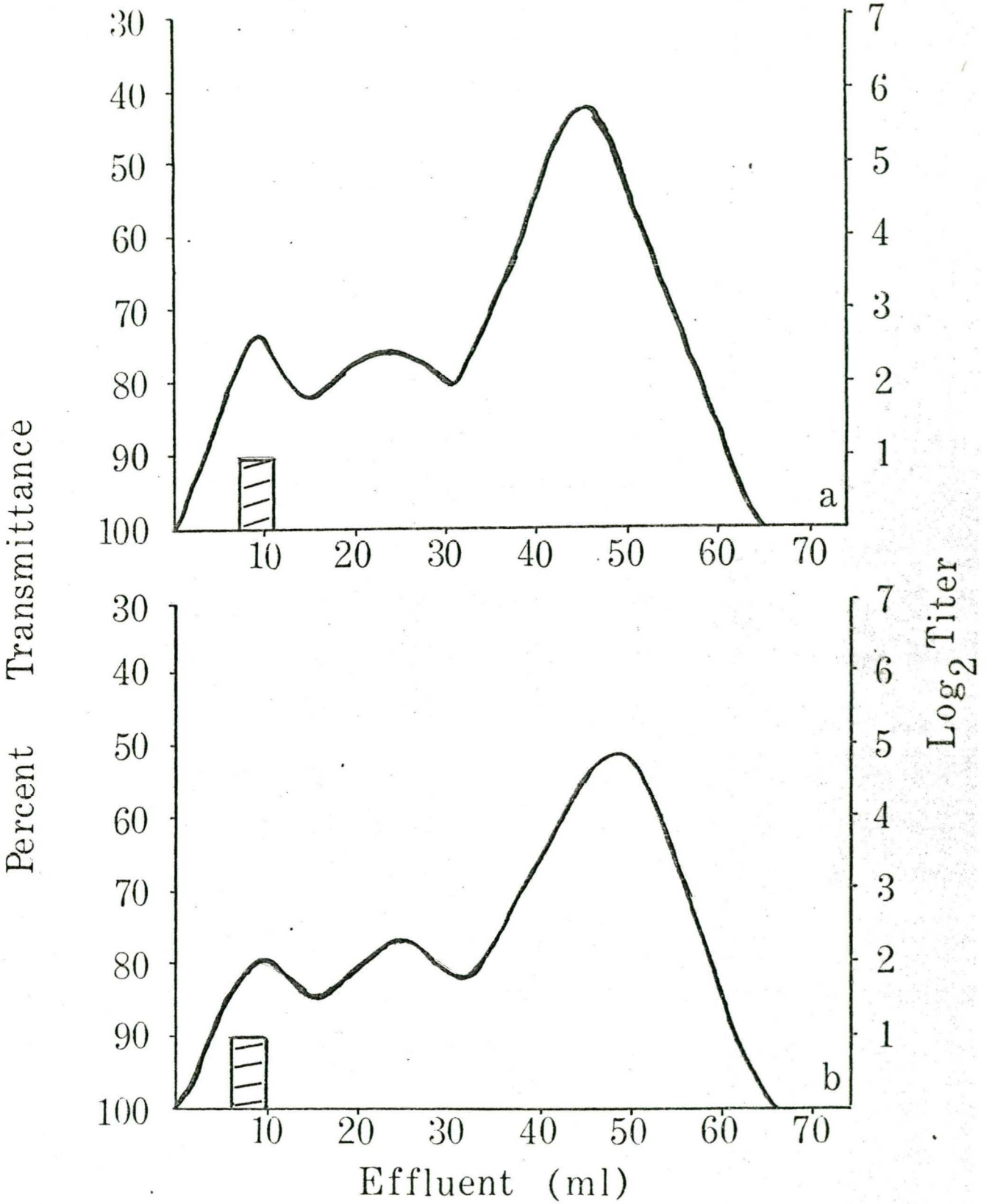

Figure 5 Fractionation of Rabbit Sera
a. Normal serum - Rab. 36
b. Normal serum - Rab. 38
Bars represent $\log_2$ titer of anti-teichoic acid antibody by passive hemagglutination.

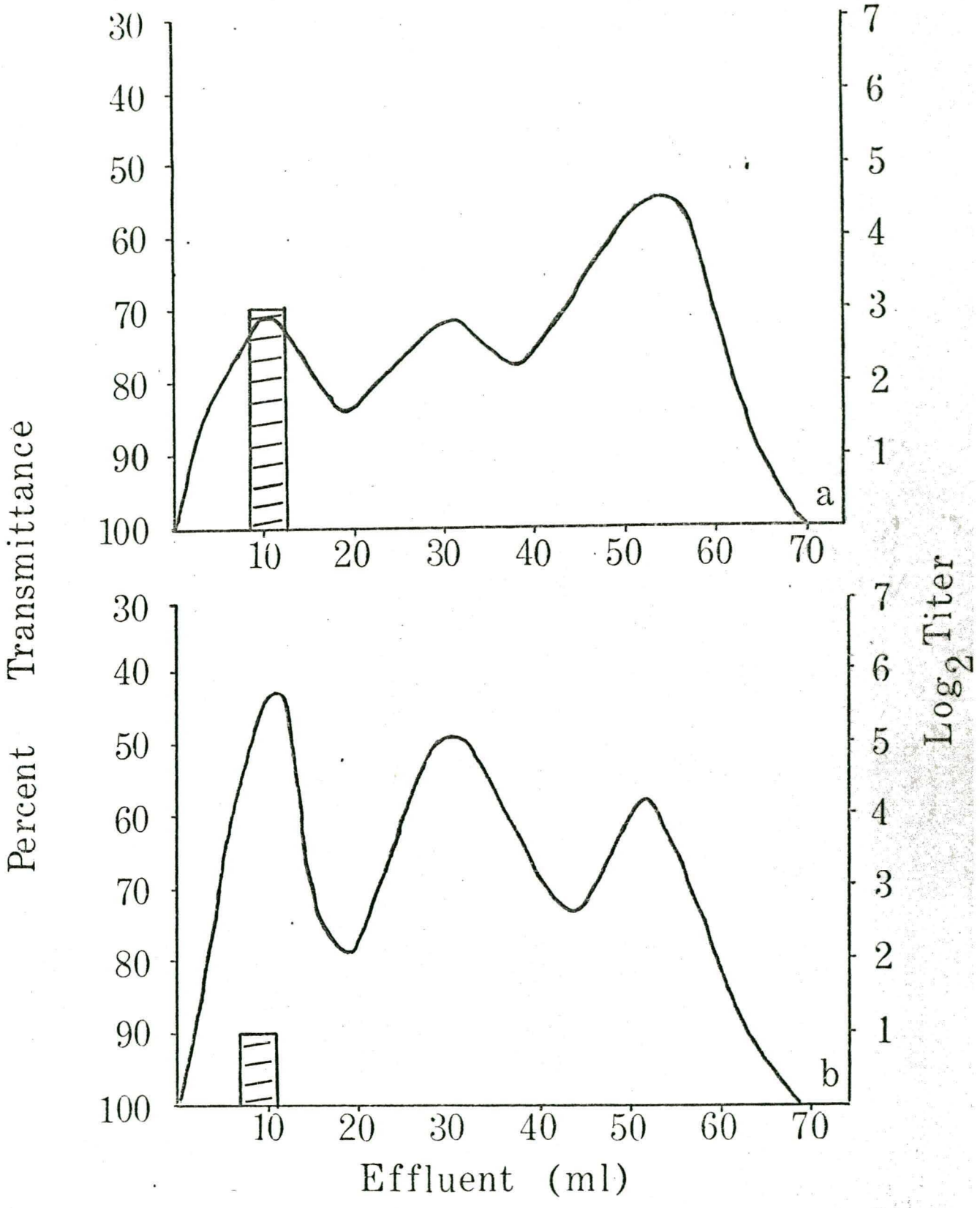

Figure 6 Fractionation of Rabbit Sera
a. Normal serum - Rab. 40
b. Four days after immunization - Rab. 41
Bars represent $\log_2$ titer of anti-teichoic acid antibody by passive hemagglutination.

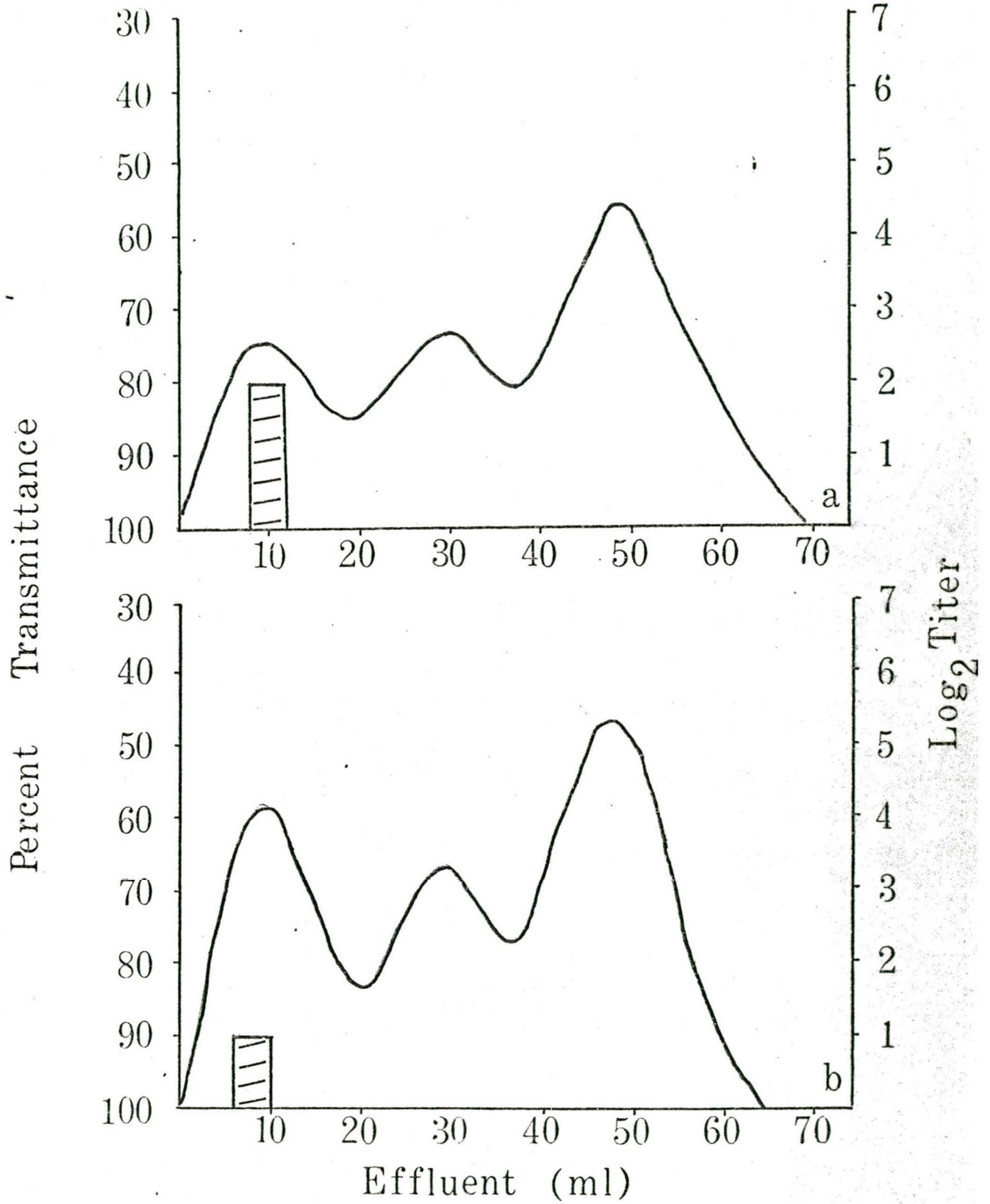

Figure 7 Fractionation of Rabbit Sera
a. Four days after immunization - Rab. 42
b. Four days after immunization - Rab. 25
Bars represent $\log_2$ titer of anti-teichoic acid antibody by passive hemagglutination.

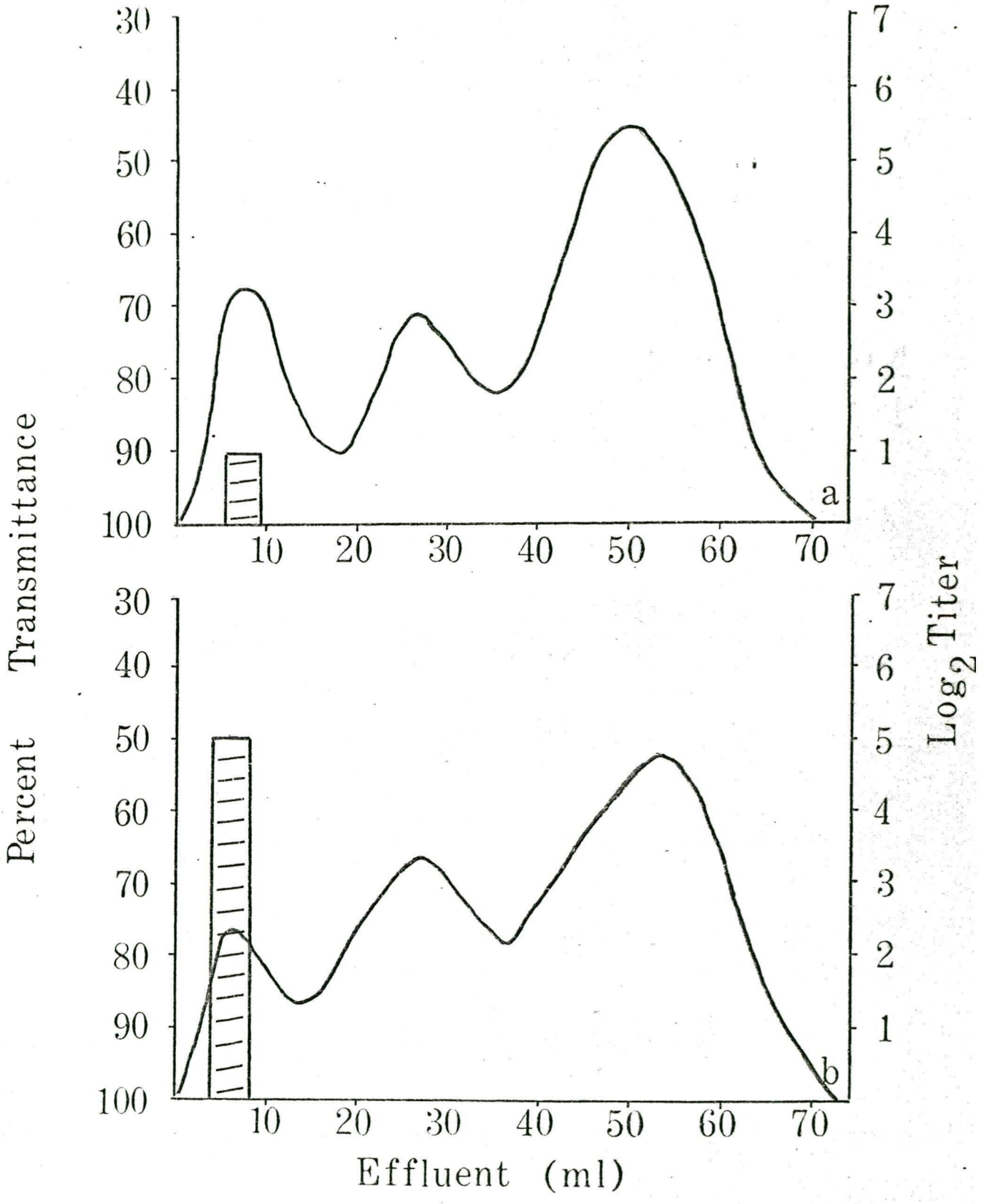

Figure 8 Fractionation of Rabbit Sera
a. Seven days after immunization - Rab. 13
b. Seven days after immunization - Rab. 43
Bars represent $\log_2$ titer of anti-teichoic acid antibody by passive hemagglutination.

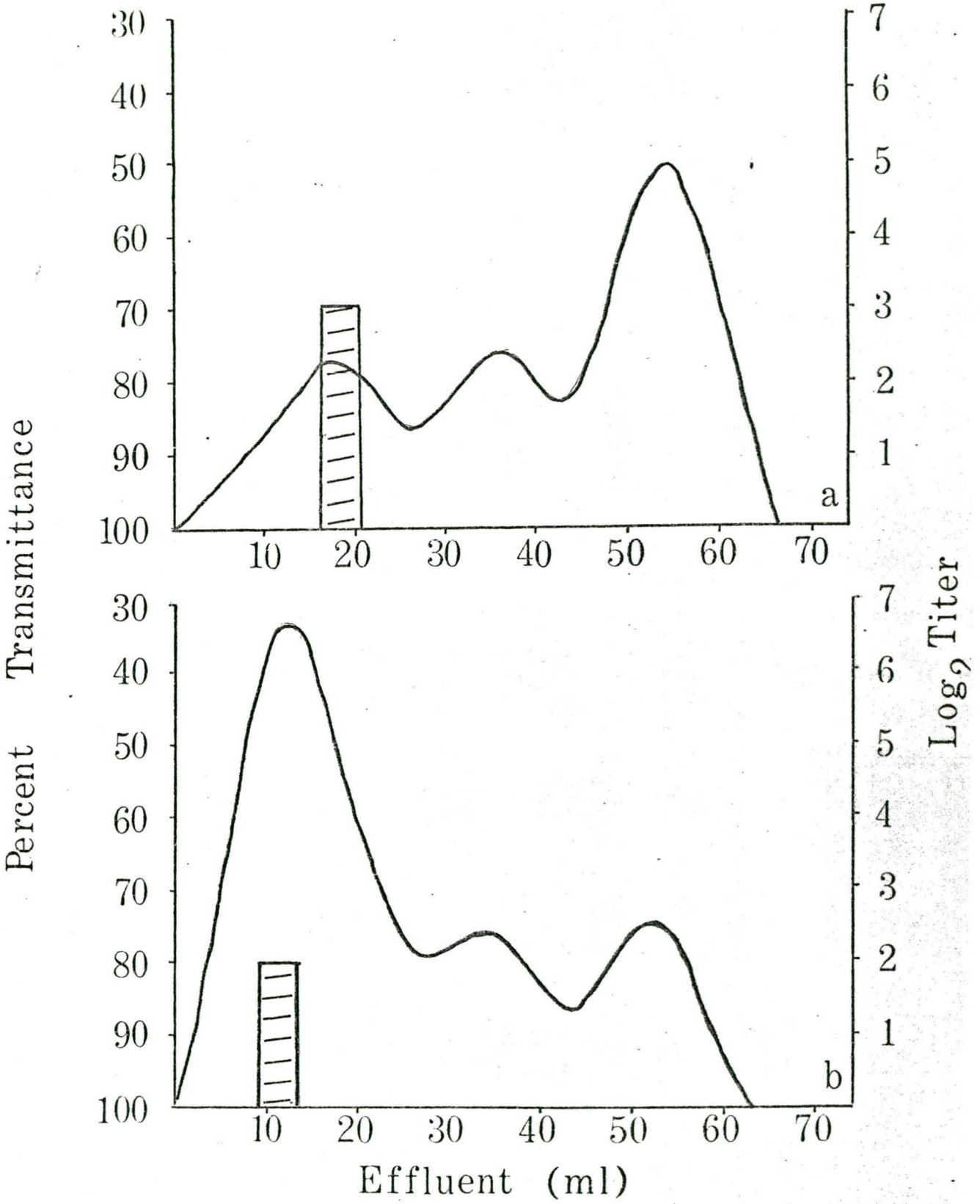

Figure 9 Fractionation of Rabbit Sera
a. Seven days after immunization - Rab. 44
b. Ten days after immunization - Rab. 16
Bars represent $\log_2$ titer of anti-teichoic acid antibody by passive hemagglutination.

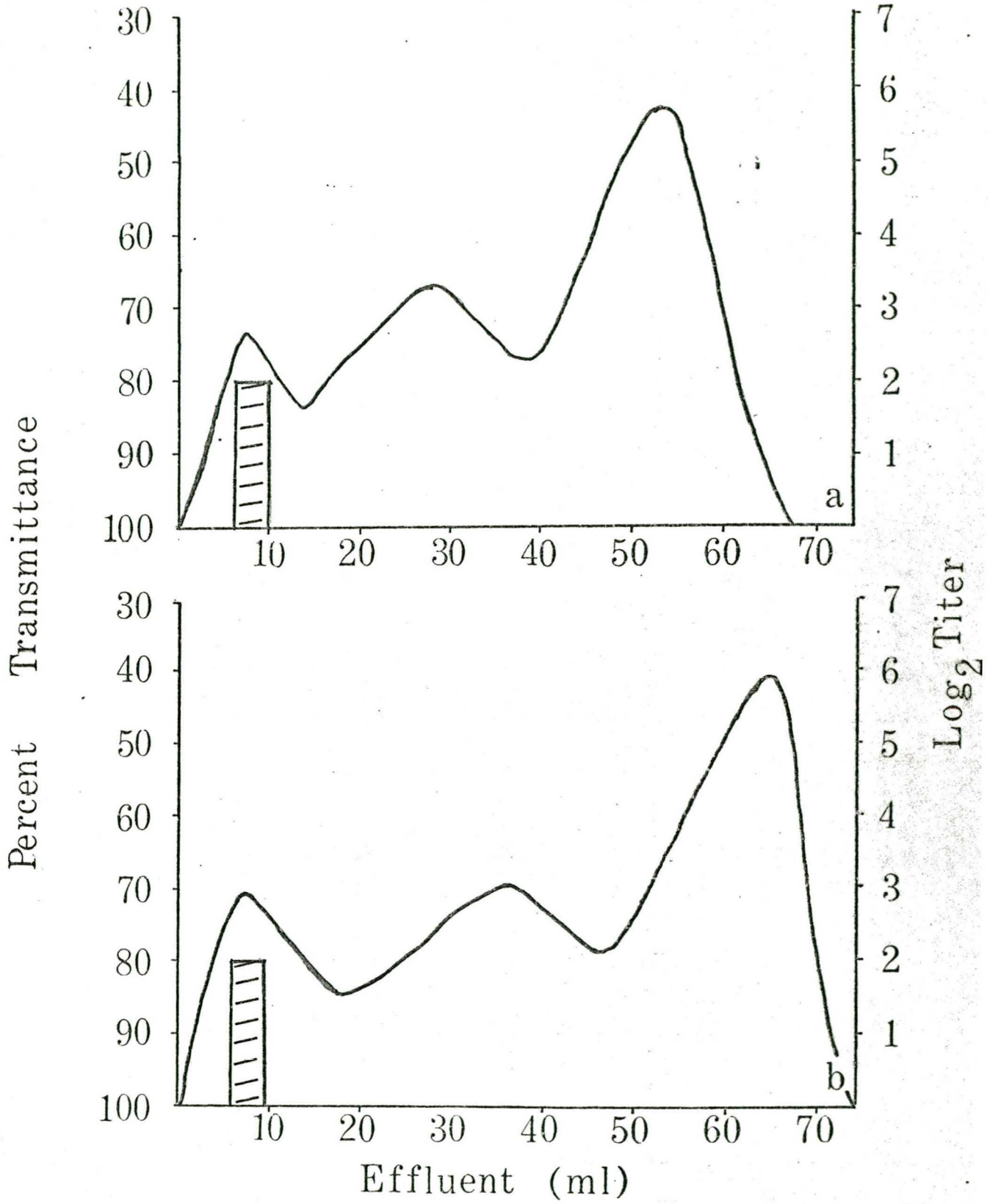

Figure 10 Fractionation of Rabbit Sera
a. Ten days after immunization - Rab. 45
b. Ten days after immunization - Rab. 46
Bars represent $\log_2$ titer of anti-teichoic acid antibody by passive hemagglutination.

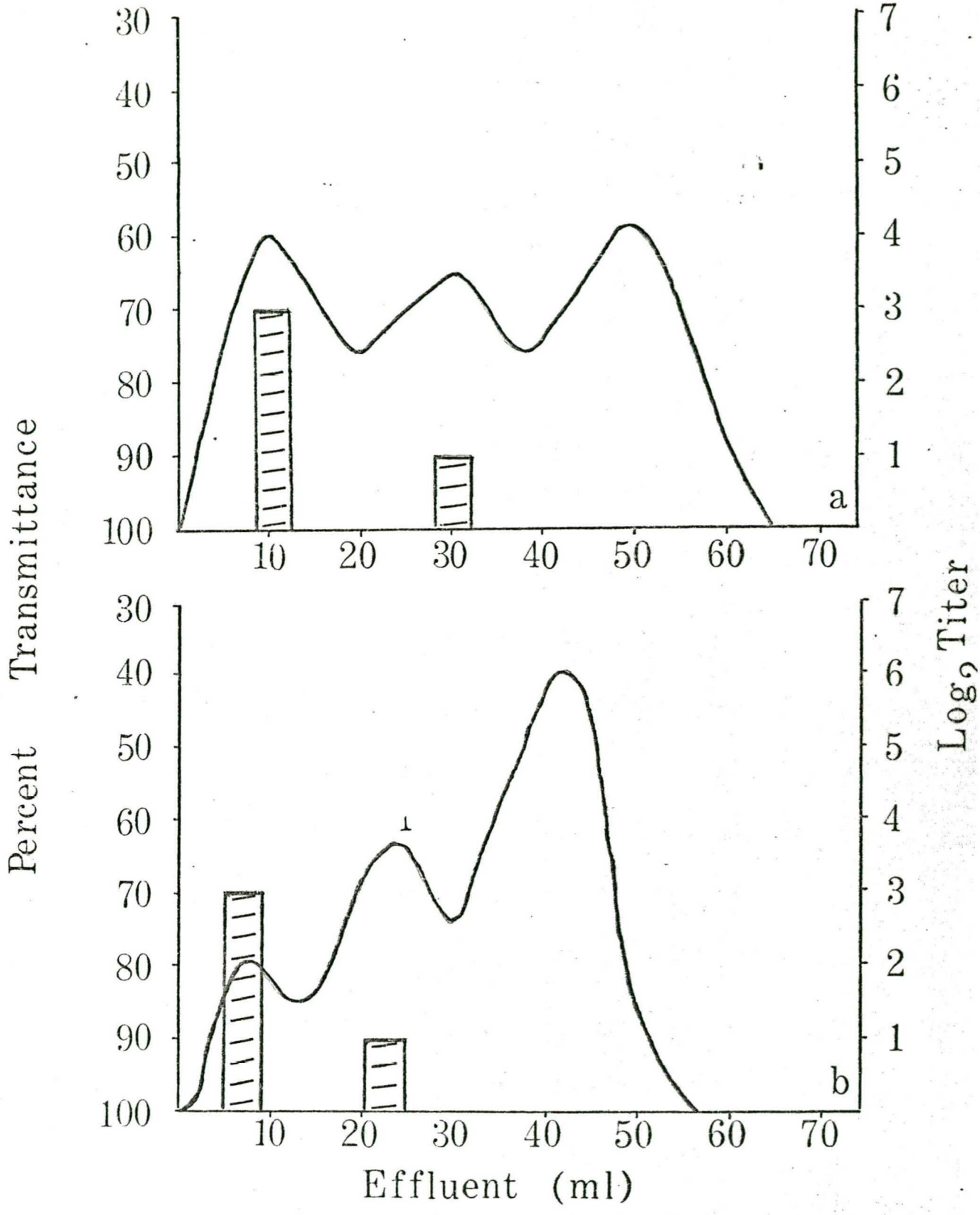

Figure 11 Fractionation of Rabbit Sera
a. Fifteen days after immunization - Rab.5
b. Fifteen days after immunization - Rab. 35
Bars represent $\log_2$ titer of anti-teichoic acid antibody by passive hemagglutination.

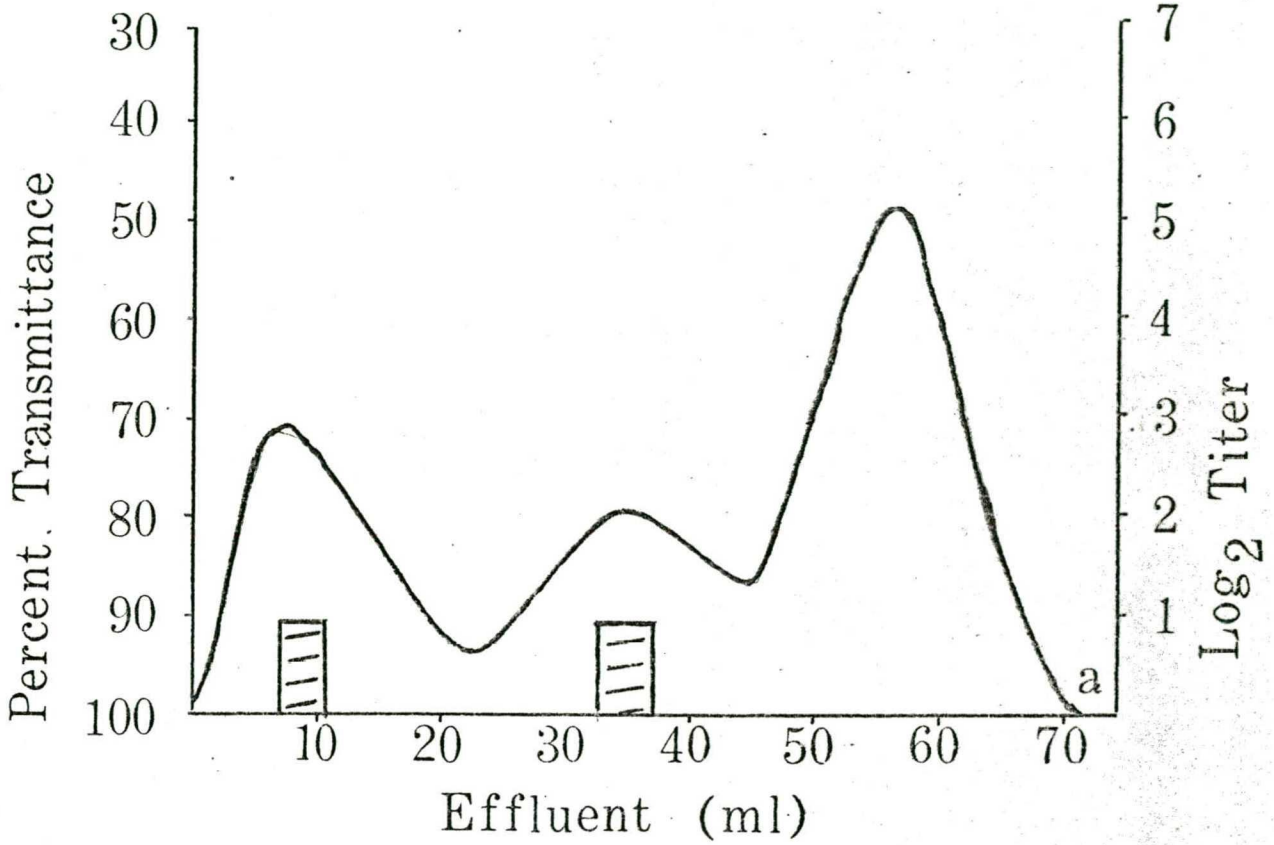

Figure 12 Fractionation of Rabbit Sera
a. Fifteen days after immunization - Rab. 27
Bars represent $\log_2$ titer of anti-teichoic acid antibody by passive hemagglutination.

Table 7

2-mercaptoethanol Treatment of Fractionated Rabbit Sera

	Titers	
	untreated	2-me treated
Rabbit 36 - normal		
Whole serum	8	0
Fraction I	2	0
Fraction II	0	0
Fraction III	0	0
Rabbit 38 - normal		
Whole serum	8	0
Fraction I	2	0
Fraction II	0	0
Fraction III	0	0
Rabbit 40 - normal		
Whole serum	8	0
Fraction I	8	0
Fraction II	0	0
Fraction III	0	0
Rabbit 41 - 4 days after immunization		
Whole serum	8	4
Fraction I	2	0
Fraction II	0	0
Fraction III	0	0
Rabbit 42 - 4 days after immunization		
Whole serum	8	4
Fraction I	4	0
Fraction II	0	0
Fraction III	0	0
Rabbit 25 - 4 days after immunization		
Whole serum	2	0
Fraction I	2	0
Fraction II	0	0
Fraction III	0	0
Rabbit 13 - 7 days after immunization		
Whole serum	4	0
Fraction I	2	0
Fraction II	0	0
Fraction III	0	0

Table 7 Continued

	Titers	
	untreated	2-me treated
Rabbit 43 - 7 days after immunization		
Whole serum	32	8
Fraction I	8	0
Fraction II	0	0
Fraction III	0	0
Rabbit 44 - 7 days after immunization		
Whole serum	8	0
Fraction I	4	0
Fraction II	0	0
Fraction III	0	0
Rabbit 16 - 10 days after immunization		
Whole serum	4	0
Fraction I	4	0
Fraction II	0	0
Fraction III	0	0
Rabbit 45 - 10 days after immunization		
Whole serum	8	0
Fraction I	2	0
Fraction II	0	0
Fraction III	0	0
Rabbit 46 - 10 days after immunization		
Whole serum	4	0
Fraction I	2	0
Fraction II	0	0
Fraction III	0	0
Rabbit 5 - 15 days after immunization		
Whole serum	8	0
Fraction I	8	0
Fraction II	2	2
Fraction III	0	0
Rabbit 35 - 15 days after immunization		
Whole serum	32	4
Fraction I	8	0
Fraction II	2	2
Fraction III	0	0
Rabbit 47 - 15 days after immunization		
Whole serum	8	0
Fraction I	2	0
Fraction II	2	2
Fraction III	0	0

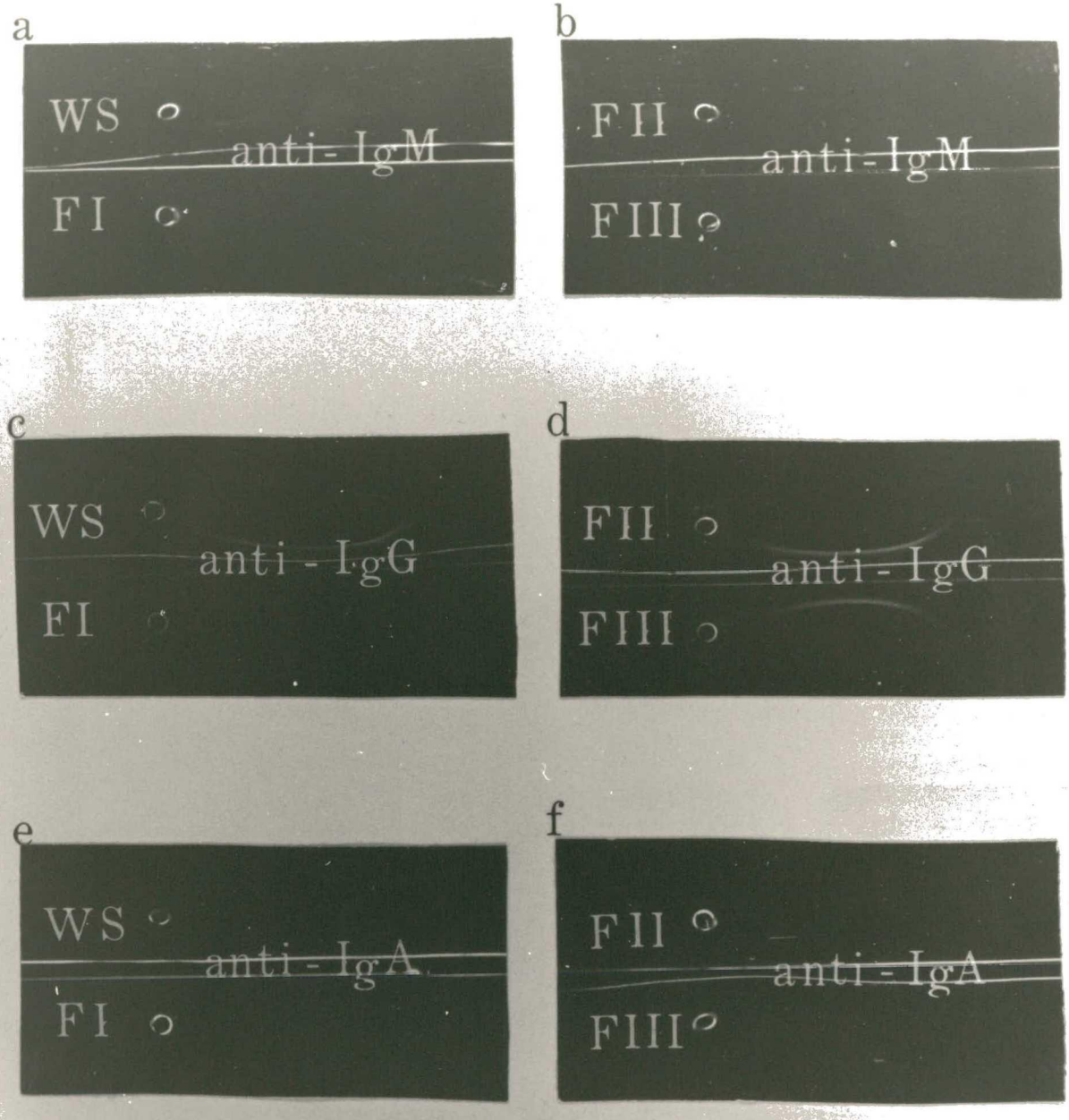

Figure 13

WS - whole serum
FI - fraction one
FII - fraction two
FIII - fraction three
anti-IgM - goat antiserum to rabbit immunoglobulin M
anti-IgG- goat antiserum to rabbit immunoglobulin G
anti-IgA - goat antiserum to rabbit immunoglobulin A

Representative Immunoelectrophoresis of Normal Rabbit Sera

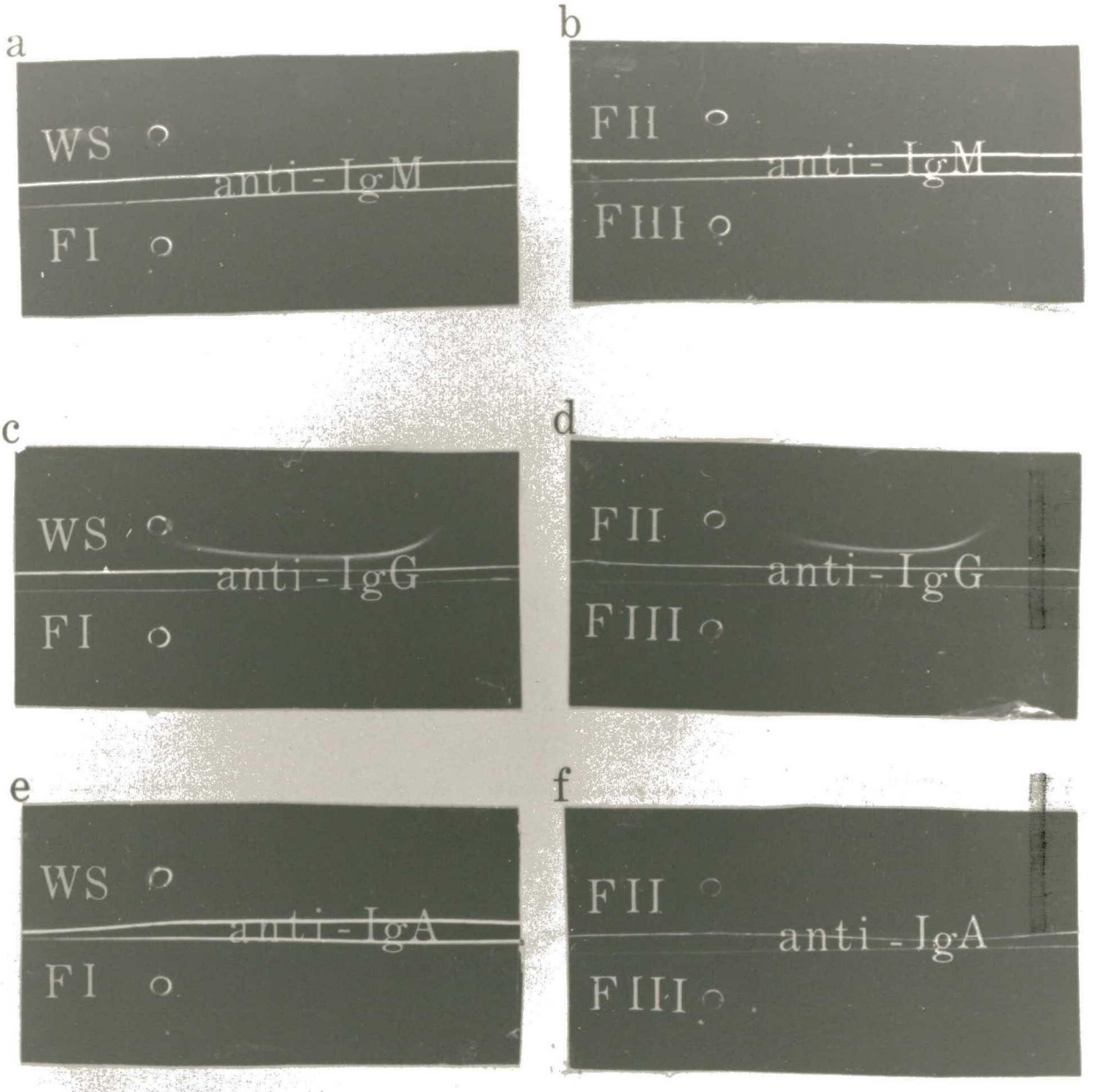

Figure 14

WS - whole serum **FII - fraction two**
FI - fraction one **FIII - fraction three**
anti-IgM - goat antiserum to rabbit immunoglobulin M
anti-IgG - goat antiserum to rabbit immunoglobulin G
anti-IgA - goat antiserum to rabbit immunoglobulin A

Representative Immunoelectrophoresis of Rabbit Sera 4 Days after Immunization

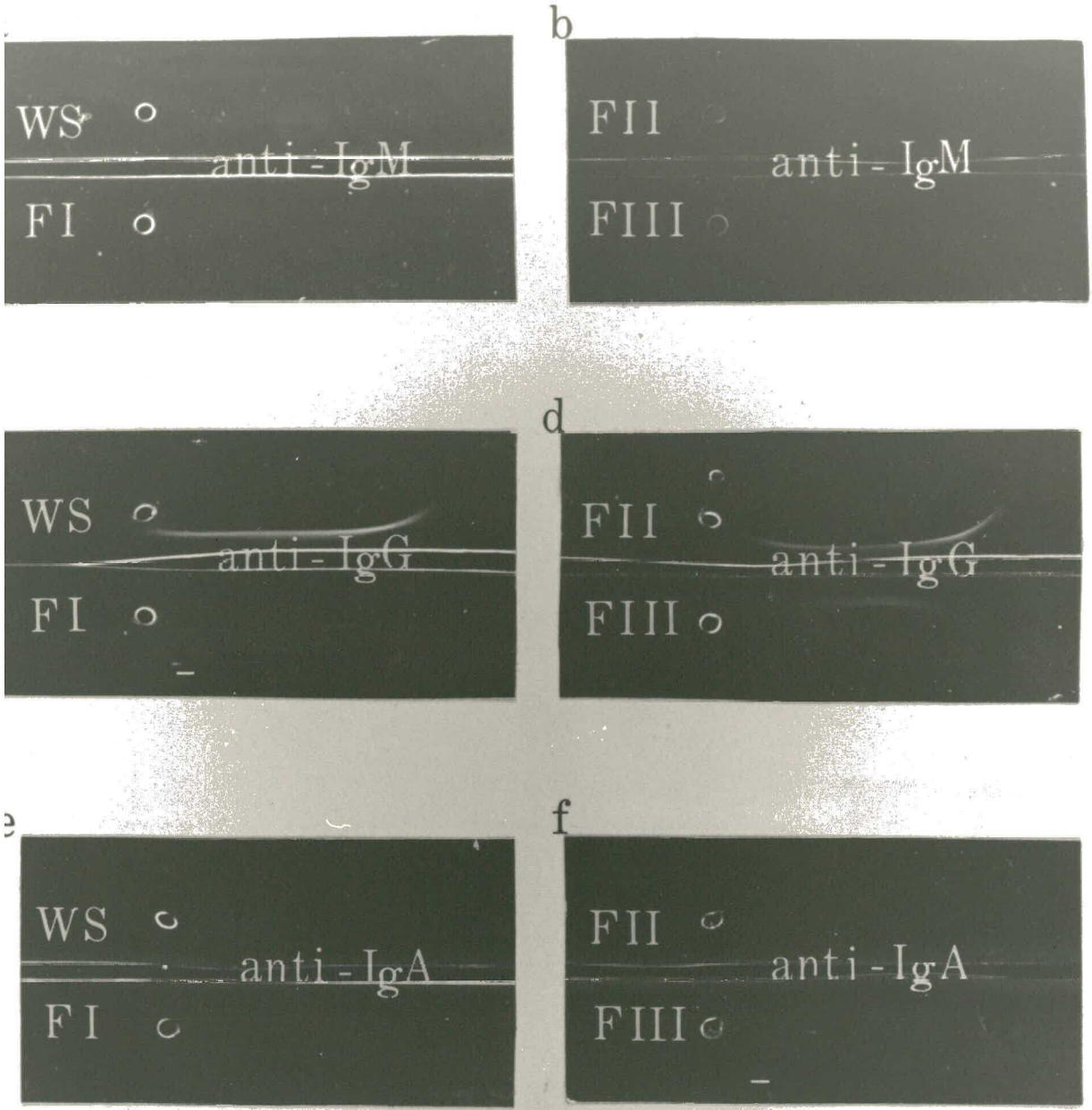

Figure 15

WS - whole serum
FI - fraction one
FII- fraction two
FIII - fraction three
anti-IgM - goat antiserum to rabbit immunoglobulin M
anti-IgG - goat antiserum to rabbit immunoglobulin G
anti-IgA - goat antiserum to rabbit immunoglobulin A

Representative Immunoelectrophoresis of Rabbit Sera 7 Days After Immunization

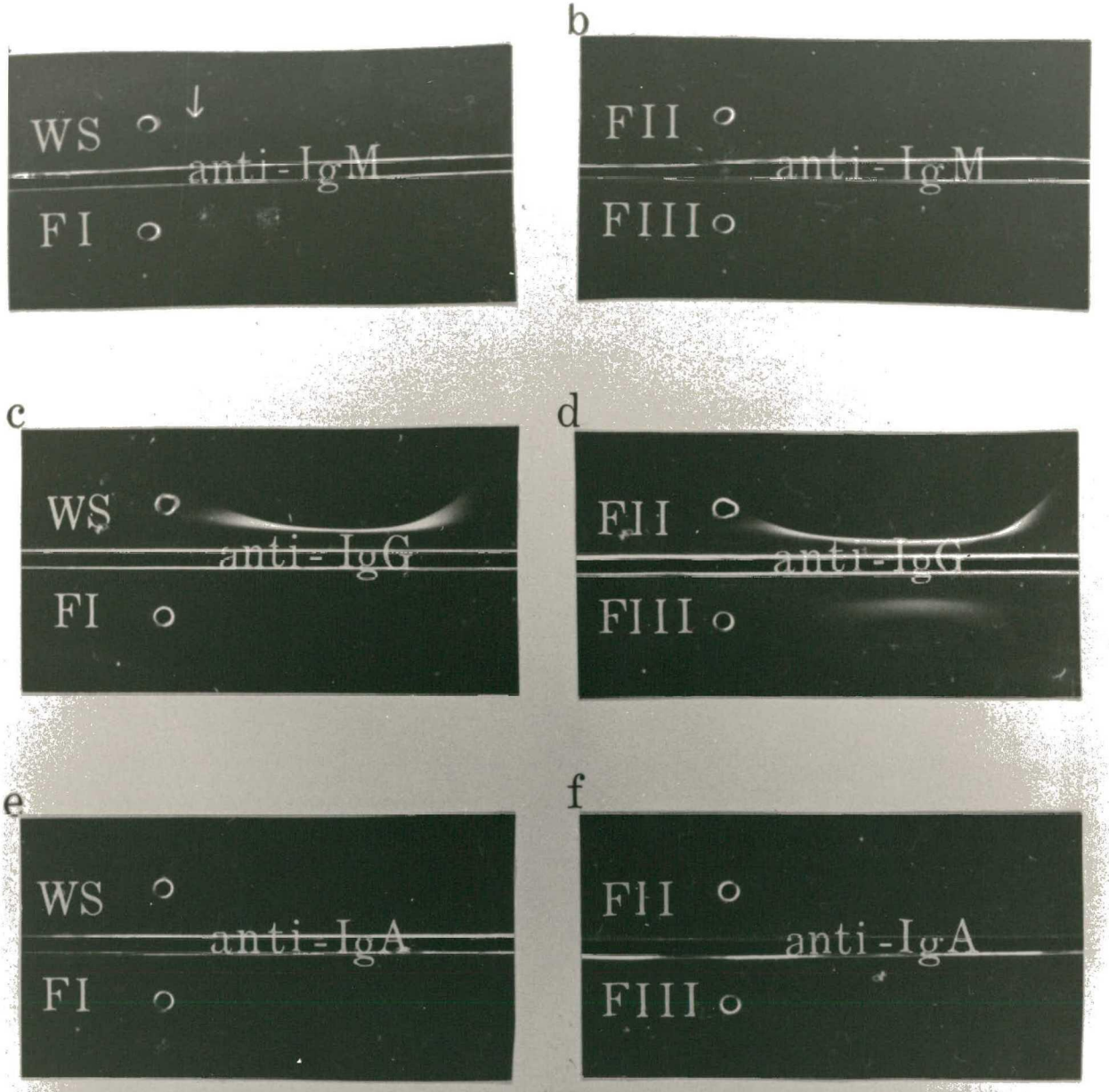

Figure 16

WS - whole serum
FI - fraction one
FII - fraction two
FIII - fraction three
anti-IgM - goat antiserum to rabbit immunoglobulin M
anti-IgG - goat antiserum to rabbit immunoglobulin G
anti-IgA - goat antiserum to rabbit immunoglobulin A

Representative Immunoelectrophoresis of Rabbit Sera 10 Days After Immunization

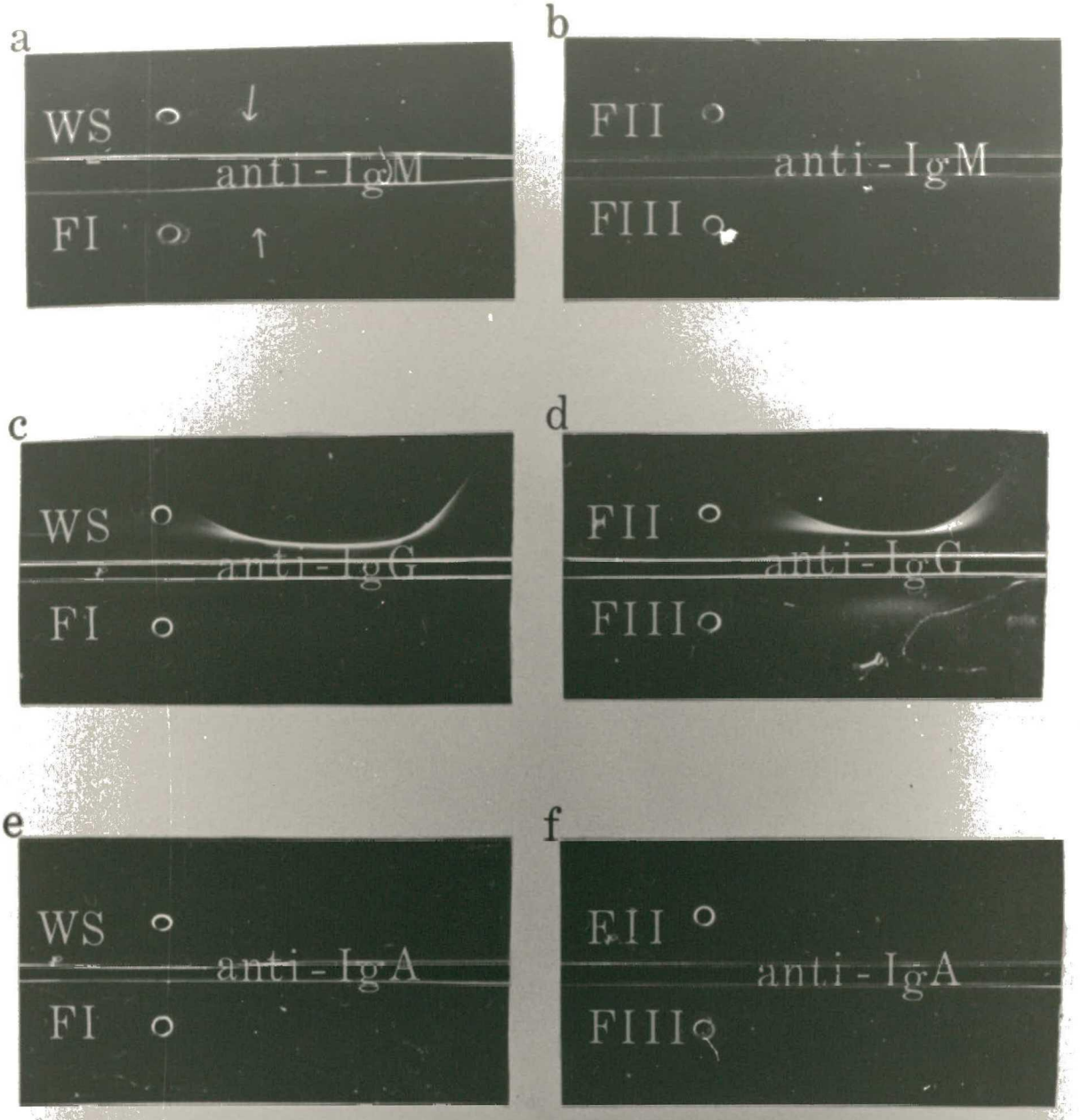

Figure 17

WS - whole serum
FI - fraction one
FII - fraction two
FIII - fraction three
anti-IgM - goat antiserum to rabbit immunoglobulin M
anti-IgG - goat antiserum to rabbit immunoglobulin G
anti-IgA - goat antiserum to rabbit immunoglobulin A

Representative Immunoelectrophoresis of Rabbit Sera 15 Days After Immunization

strated in any of the fractions or in the whole serum. Figure 18 is a comparison of the $\log_2$ titer means of the individual animals listed in Table 7. It is apparent that naturally-occurring anti-teichoic antibody in the rabbit (as demonstrated by passive hemagglutination) is 19S antibody and only after immunization is 7S antibody found.

Antibody Synthesis at the Cellular Level in the Rabbit

In normal rabbits, cells synthesizing anti-teichoic acid antibodies were observed only in the spleen. The antibodies in normal rabbits were of the IgM class, no plaque-forming cells (PFC) being observed by the indirect technique. After injection of antigen, no change in this pattern was seen until after the 4th day. Table 8 presents individual rabbit data on the numbers of direct (IgM) and amplified (IgA and IgG) plaques produced by the spleen cells from normal rabbits and rabbits sacrificed at various times after injection of the antigen. Figure 19 presents an example of the type of plaques observed by both the direct and indirect techniques. Figure 20 graphically depicts the pattern of response by spleen cells to the injection of antigen and the class of antibody released during the period. During the period studied, a maximal number of IgM plaque-forming cells were observed 4 days after immunization. However, direct plaque-forming

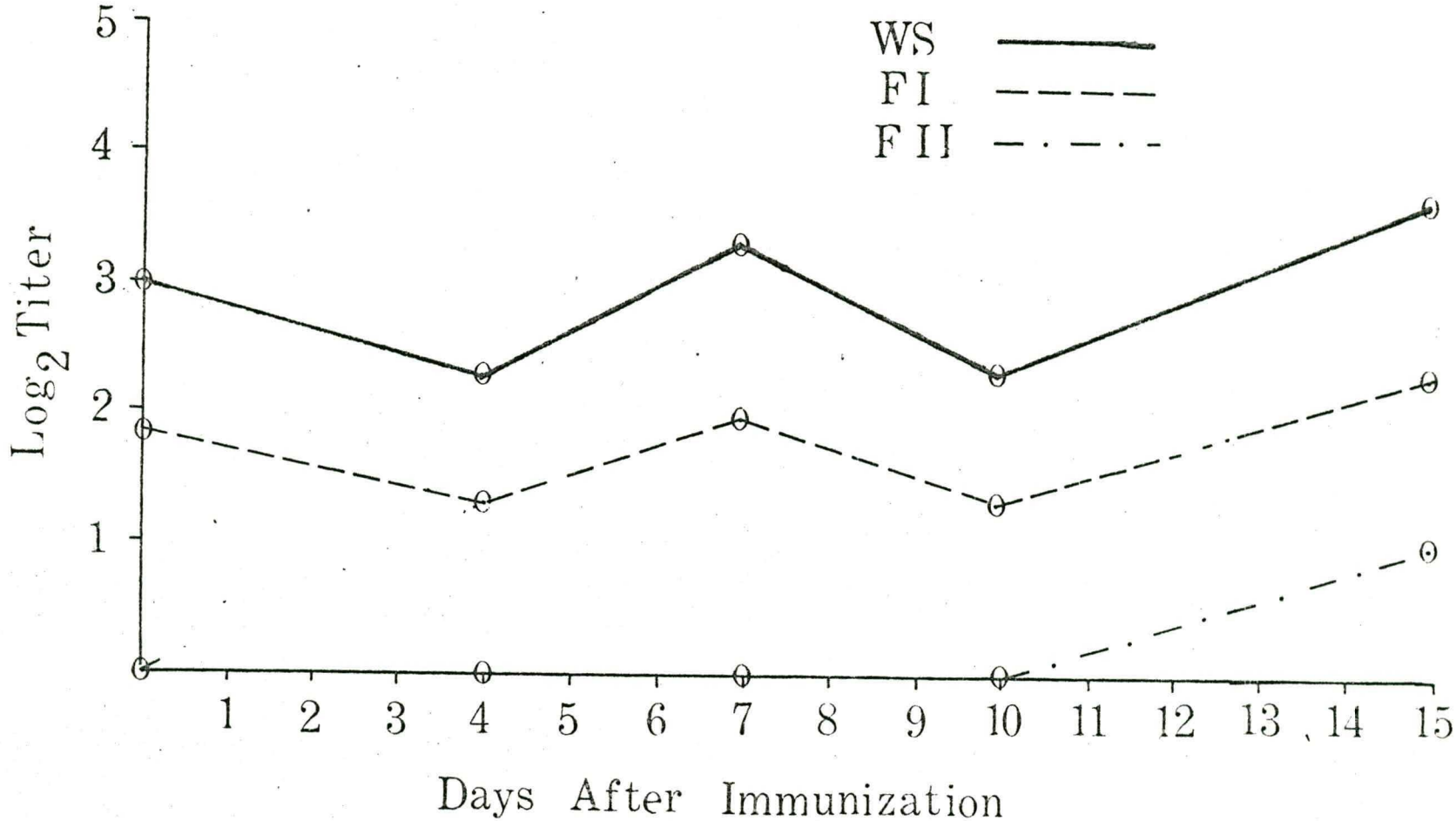

Figure 18 Comparison of Average Passive Hemagglutination Titers

Table 8

The "Primary" Response and Kinetics of the Secondary Response to Teichoic Acid in Spleen Cells of the Rabbit

Days After Immunization	Rabbit	Mean Number of PFC/10^6 Lymphocytes Direct*	Amplified** IgG	Amplified IgA
0	36	15***	0	0
0	38	11	0	0
0	40	11	0	0
4	41	30	0	0
4	42	38	0	0
4	25	31	0	0
7	43	0	2	2
7	44	0	11	0
7	13	1	6	0
8	26	6	0	0
8	39	2	0	0
8	37	3	0	0
10	27	0	0	0
10	45	0	0	0
10	46	0	0	0
15	47	0	0	3
15	48	0	0	2
15	49	0	0	0
15	5	3	0	1
15	35	2	0	4

*Direct Jerne - Mean plaques with modified erythrocytes minus mean plaques with unmodified cells

$$\text{Mean} = \text{antilog} \frac{\log (x \neq 1)}{N} - 1$$

** Amplified Jerne - (Mean plaques with modified erythrocytes erythrocytes minus mean plaques with unmodified cells) minus (Mean of direct plaques)

***Each result is based on triplicate plates for each determination.

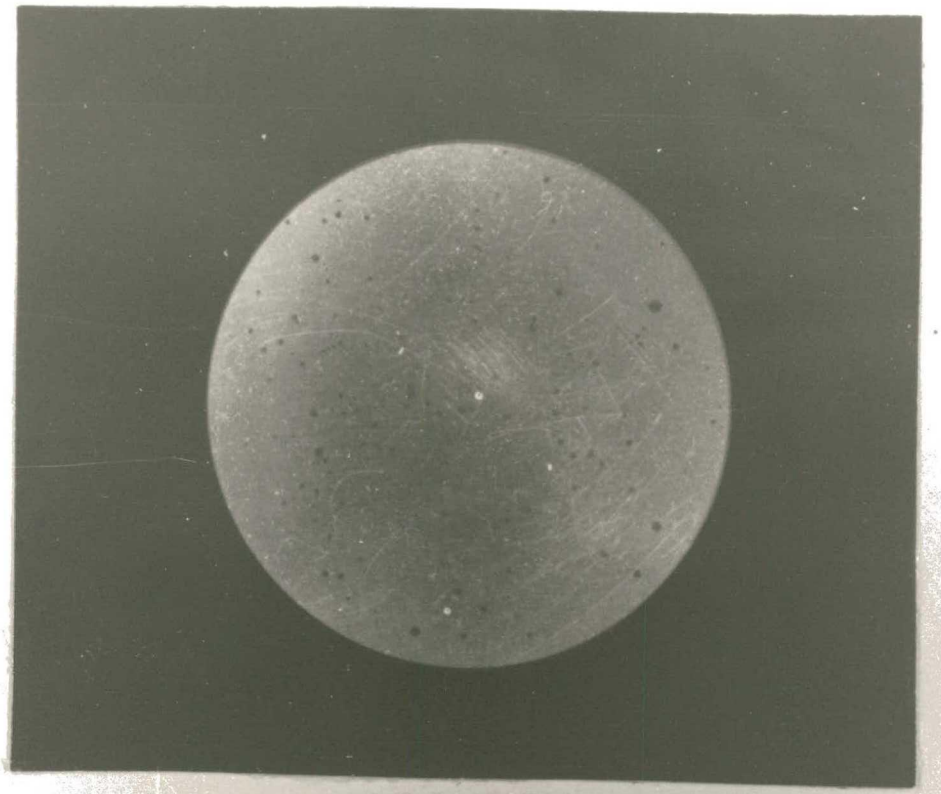

Figure 19 - a

Photograph of an Agar Plate Displaying Plaques Produced by the Antibody-forming Cells from the Spleen of a Rabbit at Four Days After Recieving a Primary Intravenous Injection of Bacillus sp. 372-56 (1 X 10^9 organisms per ml. PBS 7.3)

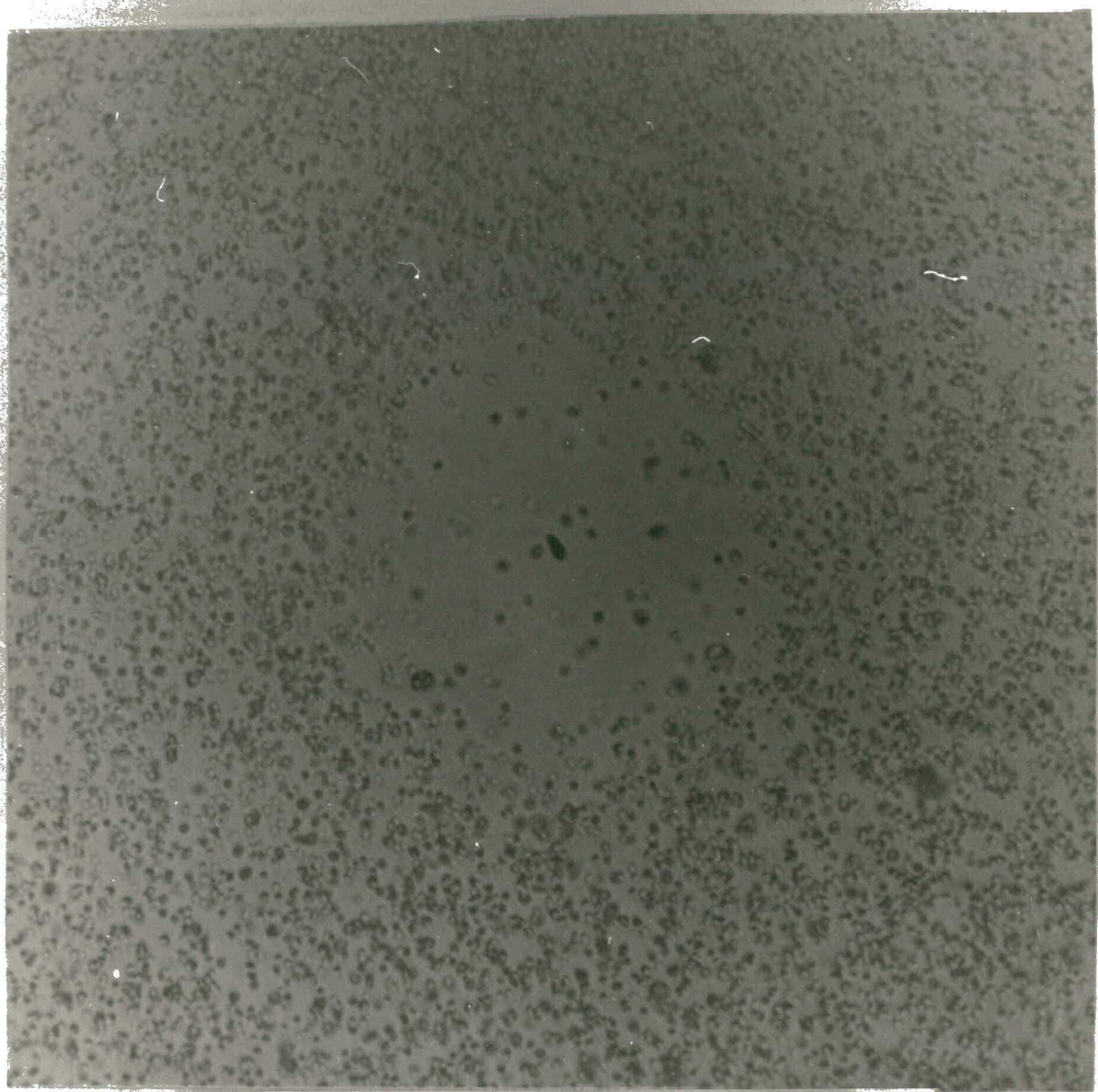

Figure 19 - b

Individual Plaque from an Agar Plate Magnified

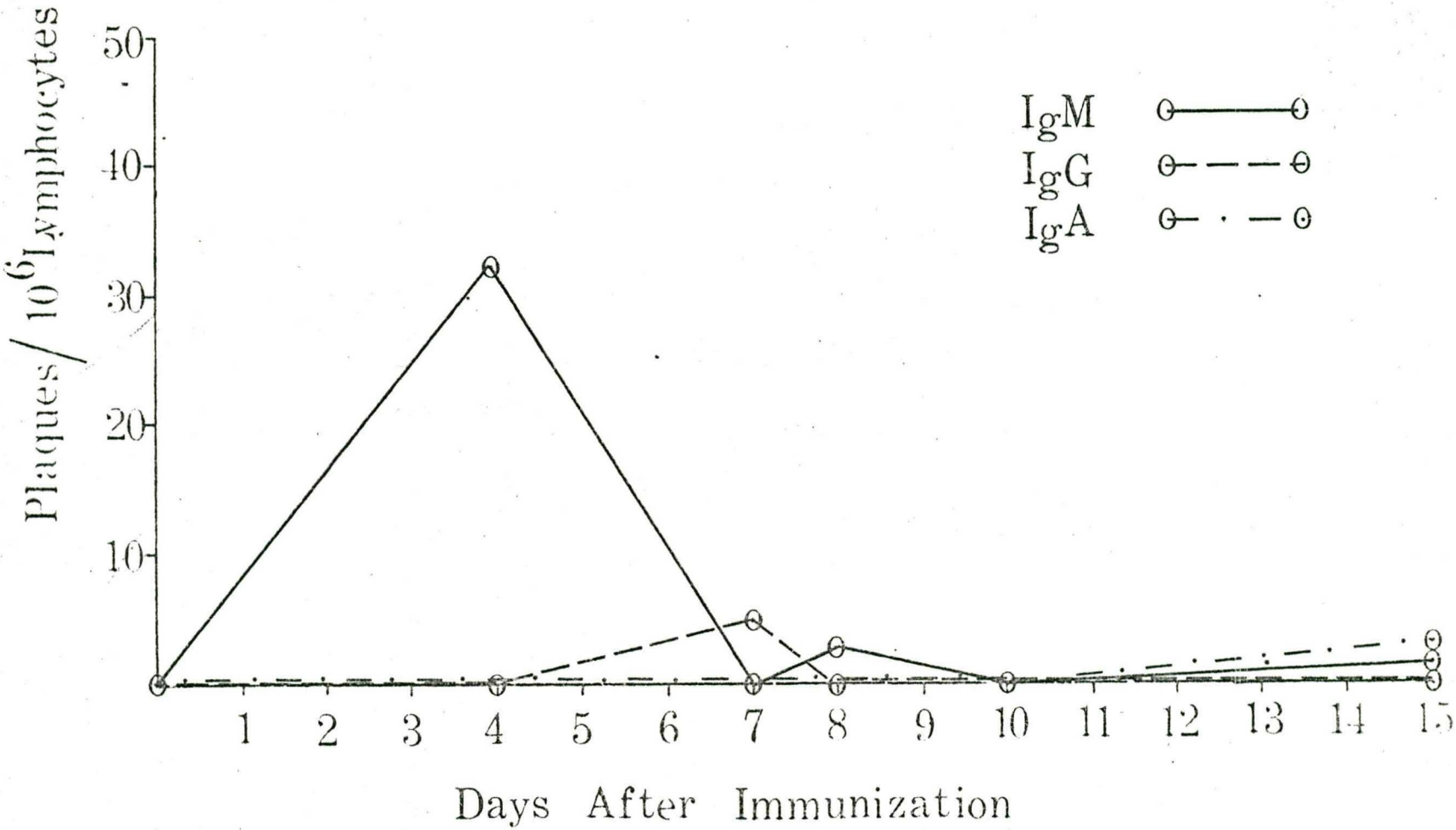

Figure 20 Kinetics and Classes of Antibody Formation by Spleen Cells in Response to the Teichoic Acid of Whole Bacilli
(Mean of Rabbits from Table 8)

cells were observed 8 days after immunization, but the magnitude of the response was much lower. After the disappearance of IgM plaque-forming cells on the 10th day after immunization, cellular activity was again observed on the 15th day. The number of amplified IgG plaques reached a maximal value at 7 days after immunization, whereas amplified IgA plaques did not appear till the 15th day after immunization. Also the number of amplified IgG plaque-forming cells was on the increase by the 15th day.

In normal rabbits, no plaque-forming cells specific for teichoic acid were observed in the mesenteric lymph nodes until after the intravenous injection of antigen (Table 9). On the 7th day after immunization, the greatest number of plaque-forming cells were observed and were limited to the IgM class(Figure 21). Neither amplified IgG nor amplified IgA plaque-forming cells were observed. Thus the times of appearance and disappearance of plaque-forming cells did not parallel those in the spleen.

Table 9

The "Primary" Response and Kinetics of the Secondary Response to Teichoic Acid in the Mesenteric Lymph Nodes of the Rabbit

Days After Immunization	Rabbit	Mean Number of PFC/10^6 Lymphocytes Direct*	Amplified** IgG	Amplified IgA
0	36	0***	0	0
0	38	0	0	0
0	40	0	0	0
4	41	0	0	0
4	42	0	0	0
4	25	0	0	0
7	43	20	0	0
7	44	25	0	0
7	13	13	0	0
8	26	0	0	0
8	39	0	0	0
10	27	0	0	0
10	45	0	0	0
10	46	0	0	0
15	47	0	0	5
15	48	0	0	0
15	49	0	0	0
15	5	0	0	0
15	35	0	0	0

*Direct Jerne - Mean plaques with modified erythrocytes minus mean plaques with unmodified cells

$$\text{Mean} = \text{antilog} \frac{\log (x + 1)}{N} - 1$$

**Amplified Jerne - (Mean plaques with modified erythrocytes minus mean plaques with unmodified cells) minus (Mean of direct plaques)

***Each result is based on triplicate plates for each determination.

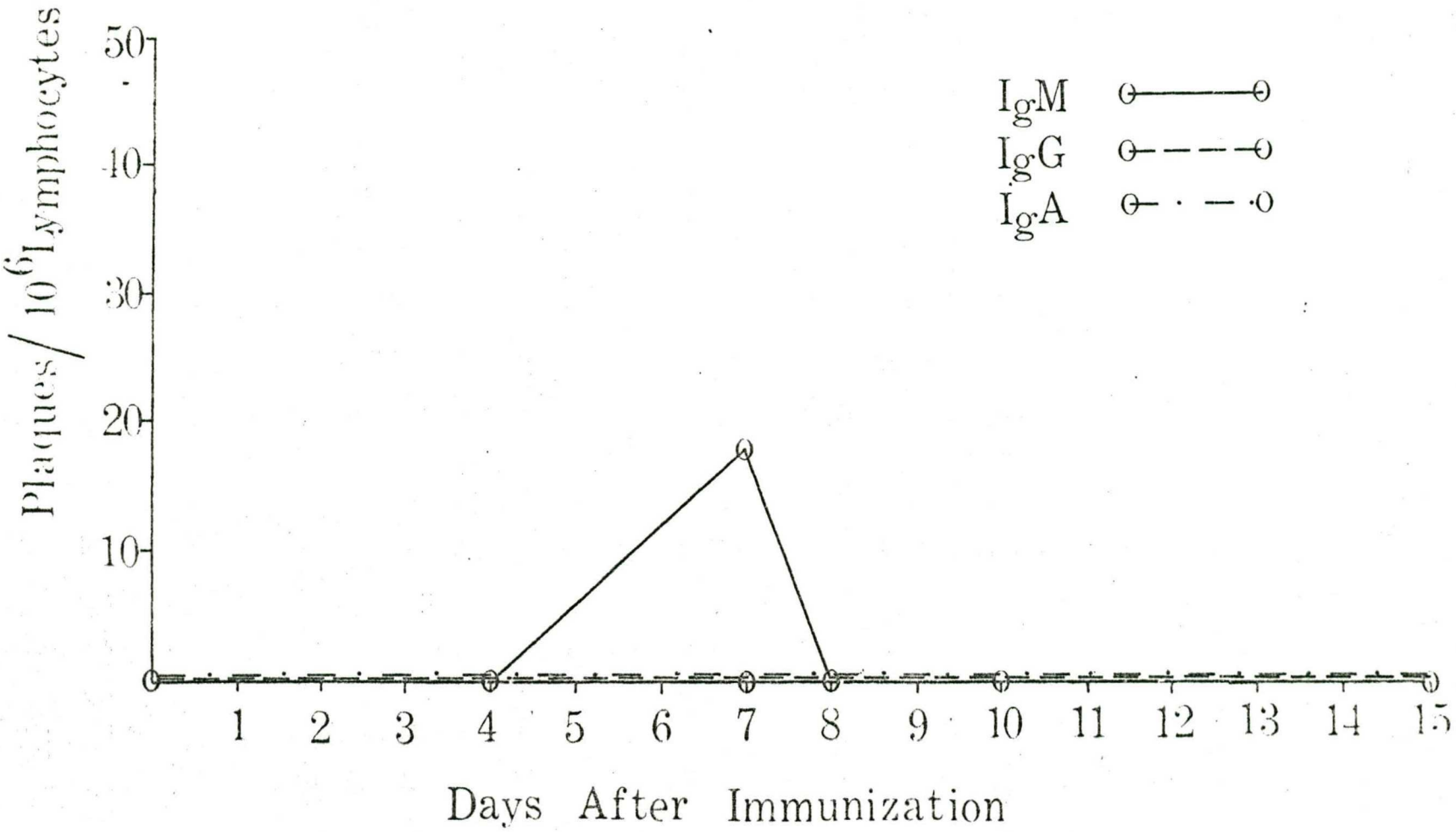

Figure 21 Kinetics and Classes of Antibody Formation by the Cells of the Mesenteric Lymph Nodes in Response to the Teichoic Acid of Whole Bacilli
(Mean of Rabbits from Table 9)

DISCUSSION

As pointed out earlier, Torii et al.(1964) and Daugherty et al.(1967) established the existence of naturally-occurring antibodies to ribitol teichoic acid in normal human sera. Natural antibodies to glycerol teichoic acid have been reported by Cherpenning and Frederick(1971), in several species, including the rabbit, but this report is the first instance of their demonstration at the cellular level.

In normal rabbits, no plaque-forming cells of the IgM, IgG, or IgA classes were demonstrated in the mesenteric lymph nodes, but direct plaques were observed in the spleen. The finding of only IgM plaque-forming cells is consistnet with the observation that anti-teichoic acid antibody from normal rabbits was detected in Sephadex Fraction I. These findings are in agreement with those of Evans(1969), who demonstrated IgM plaque-forming cells specific for a group-specific meningococcal carbohydrate antigen in the spleens of normal mice. However, since no more than one antibody-forming cell was observed per million lymphocytes, his results are statistically questionable.

The lack of observable plaque-forming cells in the mesenteric lymph nodes of normal rabbits was not expected.

The work of Frederick(1969) suggested that natural anti-teichoic acid antibodies are stimulated by a multiplicity of substances in the environment of the guinea pig with antigenic determinants corresponding to those of teichoic acid. After immunizing germ-free mice via the oral route, Bazin, Levi, and Doria(1970) demonstrated an increase in specific antibody-forming cells in the spleen and mesenteric lymph nodes. Therefore, it was assumed that if normal anti-teichoic acid antibody was due to external stimulation by the oral route, plaque-forming cells would be observed in the mesenteric lymph nodes.

After intravenous immunization with the whole organism, the response was characterized by the early appearance in the spleen of cells forming direct plaques (IgM), followed by cells producing indirect plaques(IgG) detectable only with antiglobulin serum. Again this is consistent with the appearance of anti-teichoic acid antibody in Fraction II from the sera of rabbits seven days after the appearance of IgG antibody-producing cells. This correlates with the work of others indicating peak numbers of antibody-producing cells several days previous to any observed response in the serum(Daniels and Weigle, 1967). This cellular response was similiar to that reported by others (Sterzl and Riha, 1965; Plotz et al., 1968; Wortis and Dresser, 1969; Landy et al., 1965;

Sell et al., 1970) using other antigens. Also the observation that most, if not all, antibody-forming cells following intravenous immunization first appear in the spleen is consistent with the observations of others (Jerne et al., 1963; Landy et al., 1965; Moller, 1965).

It is interesting to note the phasic appearance of 19S and 7S immunoglobulin classes of antibody-forming cells in the spleen. The phasic appearance of 7S antibody producing cells has been reported by others(Wortis et al., 1966; Eidinger et al., 1967). However, the phasic appearance of IgM plaque-forming cells is in disagreement with Sell (1970) who reported a steady decline of IgM plaque-forming cells, specific for sheep erythrocytes, after a peak response on the 4th day after immunization. The number of animals used in this investigation and the low number of plaque-forming cells observed restrict any interpretation of this cyclic variation. To further study this observation, several animals will have to be studied at each day after immunization.

It is also interesting that when direct plaques were virtually absent in the spleen, they were present in the mesenteric lymph nodes. It has been suggested by Sell, Park, and Nordin (1970) that most antibody-forming cells first appear in the spleen and that such cells are released

into the circulation to locate in other lymphoid tissues. However, in this work, it was not possible to determine if usch is the case.

Few amplified plaque-forming cells were detected in this investigation. Previously, Plotz et al.(1968) reported that the number of indirect plaque-forming cells represents a minimal number because of the presence of cells producing hemolysin that are not detected by the indirect plaque technique. It should be pointed out that the low number of plaque-forming cells detected correlates with the low antibody titer throughout the 15 day period studied.

This work has been preliminary in nature. It did point out the need for further study using not only more sensitive techniques for the detection of antibody producing cells but also studies with germ-free animals.

SUMMARY

1. A localized hemolysis in gel technique, using agarose in Gey's solution for a top layer and ion agar in Dulbecco's phosphate-buffered saline for a bottom layer, was found to be practicable for detecting antibody-producing cells in the normal rabbit.
2. Using teichoic acid modified rabbit erythrocytes as the indicator cell and rabbit anti-teichoic acid antibody, complement from various species were examined for hemolytic potential. $C'H_{50}$ titers were compared with those of a modified sheep red blood cell system using guinea pig complement. It was found that neither rat, mouse, human, or guinea pig complement could produce as much hemolysis with modfied rabbit erythrocytes as could guinea pig complement with the modified sheep cell system.
3. Humoral antibody in normal adult rabbits was characterized as a 19S (IgM) immunoglobulin specific for anti-teichoic acid. No IgG antibodies were demonstrable. These antibodies were shown to be susceptible to 2-mercaptoethanol.
4. Using a modified hemolytic plaque assay, antibody-producing cells specific for teichoic acid were

found in the spleens of normal animals. Although exposure to teichoic acid would appear to be via the oral route, no antibody-forming cells specific for teichoic acid were detectable in the mesenteric lymph nodes.

5. In the spleen, a single intravenous injection of a Bacillus species, produced IgM antibody-producing cells specific for teichoic acid on the 4th day after immunization. IgG antibody-producing cells appeared about the 7th day and decreased suddenly. IgM antibody-producing cells appeared again briefly on the 8th day after immunization.

6. In the mesenteric lymph nodes, injected animals exhibited the greatest number of IgM plaque-forming cells specific for teichoic acid 7 days after immunization. IgG and IgA plaque-forming cells specific for teichoic acid were not detected during the 15 day study period.

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