

A STUDY OF
NONPRECIPITATING ANTIBODIES

by

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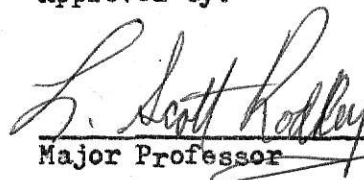
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INTRODUCTION

Immunization of animals with a foreign antigen usually results in a heterogeneous type of antibody response in which antibodies detectable by precipitation methods and those not detectable in this manner are produced. The fraction which is undetectable by precipitation methods may be found in supernatant fluids after the precipitating antibodies have been removed. Nonprecipitating antibodies are detected by various methods for assaying primary antigen binding.

Most of the previous studies of nonprecipitating antibodies have used a heterologous antigen for immunization. The primary criticism that can be made against this approach is that the nonprecipitability may be a function of the antigen instead of a unique characteristic of the antibody. If the animal responds preferentially to a determinant site that occurs only once on the surface of the antigen, there would be a significant amount of antibody that could not form the lattice structure required for precipitation.

The present investigation was undertaken to determine whether nonprecipitating antibodies would be detected as an antigen system in which the carrier proteins were made multivalent with respect to a single determinant (hapten) and were used such that only anti-hapten antibodies would be assayed. Other objectives of the study were to work out a satisfactory set of procedures to detect the presence of the nonprecipitating antibody, to purify the specific anti-hapten antibody, and to do some preliminary characterization of the nonprecipitating antibody.

LITERATURE REVIEW

There needs to be a clarification of the terms nonprecipitating antibody, blocking antibody (found in allergic situations), enhancing antibody, and anti-cytotoxic blocking factor. The reason that this needs to be done is that the literature in this area is full of confusing terms. In this study, as well as others looking at nonallergic situations, the term "nonprecipitating antibodies" refers to those antibodies which will specifically bind to their homologous antigen but which will not precipitate the antigen in the zone of equivalence, as does precipitating antibody.

The term "blocking antibody," used in allergen studies, refers to that antibody which renders the allergen unreactive to the sensitizing antibody and therefore confers some immunity to the individual. Cooke et al. (6), originally described the presence of a substance in the serum of ragweed hay fever patients treated with pollen injections that would prevent the interaction of the allergen and the sensitizing antibody. This substance was called "blocking" because of the inhibition of the interaction of allergen and sensitizing antibody. The serum activity against ragweed pollen was transferable from clinically immune treated patients to untreated patients. Another study using normal skin injected with allergen-antibody mixtures showed that a rapid reaction (1 hour) occurred if the source of antibody in the mixtures was serum of untreated hay fever patients. However, there was no reaction or only a slight reaction when the serum of treated hayfever patients was used. It was shown that this "blocking antibody" did not exert its inhibitory actions by anti-histamine effects and that the inhibition was specific, i.e., anti-ragweed pollen blocking antibody present in the serum from treated ragweed hayfever

patients inhibited the reaction of ragweed pollen and its sensitizing antibody. Cooke and others (7) have shown by electrophoretic methods that the bulk of the blocking antibody migrates with the gamma globulins. More recently, Starr and Weinstock (40) have shown that the circulating levels of blocking antibodies may be correlated with the degree of protection given the allergic patient under treatment.

The possibility of the existence of enhancing antibodies, those responsible for increasing the likelihood of tumors to survive and grow in a patient, was described as early as 1932. At that time Casey (4) reported that if suspensions of preserved tumors from rabbits that had died with Brown-Pearce rabbit tumors were injected into rabbit recipients, followed in 2 weeks by an injection of fresh tumor cells, the recipients showed a marked enhancement of tumor growth. This enhanced growth subsequently led to the death of many of the rabbits. As clearly pointed out by Medawar (28), the biological mechanism operating in immunological enhancement is different from those of acquired tolerance or immunological paralysis. The exact mechanism for immunological enhancement is not fully understood but much work has been done in attempting to elucidate this mechanism. In his excellent review of the literature, Kaliss (19) has highlighted the important aspects of the phenomenon of enhancement as known in 1958. The term "immunological enhancement" (21) was defined as successfully establishing a tumor homograft which subsequently grows, usually to the demise of the recipient, as a result of contact between the tumor and the specific antiserum of the recipient. In his review (19), Kaliss reported that there was a positive correlation between the hemagglutinin titer of an anti-tumor serum and its enhancing capacity. He suggested that the hemagglutinin (H-2) and the "enhancing" antibody were the same

moleity in an anti-tumor serum since both the agglutinating and enhancing properties were lessened by successive absorptions of the serum with red blood cells (of inbred strain indigenous to the tumor tested) and completely eliminated after absorption with the tumor tissue. At that time there were two hypotheses for a blocking mechanism to explain enhancement. One hypothesis, proposed by Medawar and his coworkers (2), was based on a distinction in mice between transplantation antigens (which can provoke resistance) and hemagglutinogens (which can evoke circulating antibodies). The two antigens were considered to both be in tissues since they were assumed to be coded for by the same histocompatibility genes. Medawar et al. (2) postulated that the tumor specific transplantation antigens were inactivated during the preparation of the tumor tissue initially injected into the recipient, leaving the hemagglutininogen present to evoke a humoral antibody response, presumably of enhancing antibody. The other hypothesis was proposed by Snell (39). He contended that the tumor homograft was fixed (walled off) by humoral antibodies, thereby keeping the tumor cells with their antigens from getting to the draining lymph nodes, the immunologically reactive centers of the recipient. In a later study by Kaliss (20) considering the possible mechanisms of enhancement, he suggested a revision of his former definition of the term enhancement. The suggested revision was "the successful establishment or prolonged survival (conversely, the delayed rejection) of an allogeneic graft." The necessity of antiserum against the graft being present in the recipient was stressed. The host must also be immunized against the graft (tumor or normal tissue). Immunization may be accomplished either passively (by injecting antiserum against the tissue into the recipient) or actively (by grafting some of the tissue onto the recipient before the test graft). If the active immunization

method is used, sufficient time must elapse between initial graft and test graft to prevent a "second set" rejection (accelerated rejection of a second graft). In his study of strain A tumor Sarcoma I in mice, Kaliss (20) concluded that at least three things were of importance in the mechanism of enhancement. The first of these was that there was a qualitative change in the type of host cells operation against a first or second set graft. The second was that presence or absence of vascularization of the graft was a contributing determinant in enhancement development. The third was that there was a combining of action between humoral antibody and host cells that might result in either enhancement or in graft destruction. In their three studies on the subject of enhancing antibody, Zimmerman and Feldman (46, 47, and 48) reported their studies using normal skin grafts in rats. They passively immunized neonatal Lewis rats (given enhancing antisera made in adult Lewis rats after grafting skin and injecting spleen cells of the same strain of rats that the test grafts were to be from) at the same time they have test grafts. They found that the viability of the skin grafts was significantly prolonged. They also found that the enhancing property of the antiserum resided in the IgG fraction and that it was specifically effective for the grafts of the immunizing strain (46). The second of their studies (47) showed that the enhancing activity may be not totally specific but that there can be a degree of cross-reactivity between two strains of rat donor skin grafts. Although there was some cross-reactivity, there was better enhancing ability of each serum used with its homologous skin graft. In the third report (48) they showed that skin grafts of rats dipped in the appropriate enhancing antiserum were kept longer than undipped grafts. This was interpreted to mean that enhancing antibodies were active peripherally in the skin graft or its draining lymph

node. No direct evidence was obtained for binding of enhancing antibodies to the homograft. However, in a later study by Jones, Peter, and Feldman (17), specific binding of enhancing antibodies to target skin grafts within two days was observed by using ^{125}I radiolabelled enhancing antibodies.

Recently much attention has been focused on the blocking of cytotoxic effects of immune lymphocytes mediated by a humoral product. Takasugi and Klein (41) used enhancing mouse antiserum to study the possible blocking mechanisms involved in in vivo and in vitro tumor enhancement. They employed the microassay for cell-mediated immunity to evaluate immune lymphocytic cytotoxicity against target cells. They found that two types of block seem to be operating during enhancement. One of the blocks involves protection of target tumor cells from immune cells by antibody. A block earlier than that involved after the production of the above protecting antibodies was indicated when they examined the lymph node and spleen cells of those animals injected with antiserum as well as tumor cells. They found that those lymphoid tissues showed weaker immunity than did the lymphoid tissues of those animals given only tumor cells. They also observed that the IgG1 and IgG2 fractions of the enhancing antiserum were active both during in vivo enhancement of the tumor and during in vitro blocking of the lymphocytic cytotoxicity toward the tumor cells. Studying induced mouse sarcomas, Sjogren, et al. (36) found that the sera of those mice with progressively growing tumors could block the cytotoxic affects of lymphocytes that were specifically immune for the type of neoplasm that the mouse had. The blocking activity could be absorbed from the sera using the respective tumor cells and could be eluted from these cells at low pH (3.1). A low (less than 10^5 daltons) and a high (greater than 10^5 daltons) molecular weight fraction could be obtained from the eluates. Neither of the

above fractions by themselves could inhibit cytotoxicity although a 1:1 ratio of each fraction could inhibit following incubation with target cells before adding immune lymphocytes. Their study also indicated that the low molecular weight fraction and the 1:1 mixture of both fractions, but not the high molecular weight fraction, could block if mixed with immune lymphocytes prior to adding them to target cells. It was postulated that the blocking factor seen in these mouse sera was an antibody-antigen complex which could bind to target cells and/or react with lymphocytes immune to their antigens. The latter possibility was thought to be temporary. Sjogren and Borum (35) found, in their study of rats with polyoma and Rous sarcomas, that blocking activity against the cytotoxic effects of lymphocytes specifically immune to the antigens of the respective tumor was present in sera from the majority of those animals with tumors. Those rats that did not develop primary tumors had no detectable blocking activity in their sera. In 1972 Sjogren and coworkers (37) reported that "blocking factors" could be eluted from human tumor tissues at pH 3.1. These results parallel those reported earlier in the mouse tumor studies (36). Again both a high and a low molecular weight fraction were separated from the eluate. Neither fraction could cause blocking by itself, but a 1:1 ratio was effective. Blocking also resulted when the tumor cells were first exposed to the high molecular weight fraction and then to the low molecular weight fraction. The reversed sequence resulted in no blocking. An interesting ramification of these studies is the report by Levy et al. (24). They studied the blocking activity of the serum of a patient with melanoma, who, during the course of treatment, was given an injection of Bacillus Calmette-Guerin (BCG). His lymphocytes showed cytotoxicity for the autogenous tumor cells both before and after the injection of BCG. Although the pre-injection serum

showed no blocking activity, the post-injection serum showed activity. This blocking activity was seen in association with clinical deterioration. It was emphasized that this one study should not contraindicate the use of BCG in all tumor therapy but that it does indicate that there may be some deleterious effects.

Although nonprecipitating antibodies have been observed in several species, they were first observed by Heidelberger and Kendall (15) in 1935 while they were expanding their previous work on the quantitative precipitin test. They used crystalline egg albumin as the antigen to inject rabbits. They observed that the antibodies made to the egg albumin were not homogeneous but contained both precipitating antibodies and some antibodies that would not precipitate with antigen by themselves but would coprecipitate with the precipitating type of antibodies. These nonprecipitating antibodies appeared to be in the sera of the rabbits even after prolonged immunization.

In 1949, Kabat and Benacerraf (18) showed that nonprecipitating rabbit anti-ovalbumin antibodies were just as effective per unit weight as the heterogeneous anti-ovalbumin antibodies present in the whole serum in producing passive anaphylactic sensitization in guinea pigs. In this study the precipitating antibodies were removed by serially absorbing the antiserum with antigen. The nonprecipitating antibodies were found in the supernatant after the serial absorption. The amount of nonprecipitating antibody was quantitated by adding calibrated amounts of antiserum and antigen to the supernatant to make the nonprecipitating antibodies coprecipitate. The passive sensitization of the guinea pigs was done by injecting dilutions of antiserum intravenously which contained known amounts of nonprecipitating antibody. Two days later the guinea pigs were injected

intravenously with ovalbumin and the degree of anaphylactic reaction was recorded. They postulated that the nonprecipitating antibodies seen could be univalent and still cause the observed anaphylactic activity if they were present in sufficient quantity.

Nonprecipitating antibodies that were made in rabbits injected subcutaneously with either bovine serum albumin (BSA) or human serum albumin (HSA) emulsified in complete Freund's adjuvant were studied by Feinberg (10) in 1958. He used a modification of the quantitative precipitin test, using radioactively iodinated BSA (or HSA) as the antigen, to test the precipitating capacity of the antiserum. He found that there was an appreciable amount of iodinated antigen that remained in the supernatant in the zone of antibody excess with some of the sera tested. Up to 95% of the iodinated antigen in the supernatant was precipitated by sheep anti-rabbit globulin antiserum. To show that this was due to specific antibody bound to the iodinated antigen and precipitated by the anti-globulin antiserum, some of the iodinated antigen in the supernatant was mixed with rabbit anti-egg albumin and precipitated by the sheep anti-rabbit globulin antiserum. This resulted in the precipitation of less than 3% of the iodinated antigen. Paper electrophoresis was used to monitor the supernatant after precipitation of unlabelled rabbit anti-human serum albumin with ^{131}I -HSA in the zone of antibody excess. The major radioactive peak migrated as a gamma globulin, suggesting that antibody was bound to the ^{131}I -HSA and that the nonprecipitating antibody was a slowly migrating gamma globulin which had its mobility slightly increased by being bound to the faster migrating HSA. Another group of rabbits, injected with alum precipitated antigen, produced a negligible amount of nonprecipitating antibody. The time of appearance of the nonprecipitating antibody for those rabbits injected with antigen

emulsified Freund's adjuvant was 8-16 weeks, with the highest amount being at 12 weeks. These nonprecipitating antibodies were not thought to be the coprecipitating type that were described by Heidelberger (15) because there was no decrease in nonprecipitated label if precipitation was done at the zone of equivalence instead of in antibody excess.

Fiset (12) in 1962 found that rabbit nonprecipitating antibodies against egg albumin and bovine plasma albumin were not active agglutinins as assayed by the tannic acid hemagglutination technique. The separation of precipitating and nonprecipitating antibodies was done by serial absorption of the antisera with small amounts of antigen and was considered to be complete when a negative ring test resulted. Agglutination occurred with just the supernatants obtained by the above absorption technique and also occurred after the nonprecipitating antibodies had been coprecipitated. Therefore, an agglutinin, separate from the nonprecipitating antibody was thought to be the active agent in demonstrating agglutination. In contrast to the findings of Feinberg (10), Fiset (12) showed that nonprecipitating antibodies could be made by rabbits injected with alum precipitated antigen. However, nonprecipitating antibodies were not detected in those rabbits injected with soluble antigen. She also observed that nonprecipitating antibodies were able to delay but not inhibit the combination between the homologous antigen and the precipitating antibody.

Anti-azo benzene sulphonate precipitating and nonprecipitating antibodies were studied by Carter and Harris (3) in 1967. The nonprecipitating antibodies were obtained by serially absorbing the rabbit sera with supernatants onto a solid immunoadsorbent. They found that the nonprecipitating antibodies had a greater electrophoretic mobility than the precipitating antibodies. The precipitating antibodies were identified

as IgG molecules and the nonprecipitating antibodies were thought to be IgA molecules. They thought that there were more negative ionizable groups on the nonprecipitating antibodies because of the greater electrophoretic mobility of the nonprecipitating antibodies as compared to the precipitating antibodies. They postulated that this higher negative charge could be an important cause of nonprecipitation.

Characterization of nonprecipitating antibodies in antiserum from rabbits injected intravenously with soluble BSA or HSA was reported by Christian (5) in 1970. A few of the rabbits had no demonstrable precipitins in their sera, yet had antigen binding activity, as assayed by the Farr technique. In characterization studies done to compare precipitating with nonprecipitating antibodies, the two types of antibodies were similar in their electrophoretic mobilities and in their DEAE Sephadex chromatography properties. These nonprecipitating antibodies demonstrated a reaction with the respective antigen in the hemagglutination technique (coprecipitation was not done to see if the activity remained in the supernatant as the results of Fiset (12) indicated), and were active in passive cutaneous anaphylaxis and complement fixation (they were actually more efficient at complement fixation than the same concentration of precipitating antibodies). One possible explanation for the observed nonprecipitability of these antibodies, given by Heidelberger and Kendall (15), was that more precipitating activity may be seen later in the course of immunization with complex antigens (such as BSA or HSA) because antibody with specificity toward more of the determinants on the complex molecules was made later. Christian's (5) hypothesis was that a number of the rabbits were partially tolerant to the determinants on the multideterminant antigen (BSA or HSA) and subsequently recognized and gave an antibody response to

only a few of them, too few to give the complex lattice structure required for precipitation.

The properties of nonprecipitating antibodies were also studied by Margni and Bineghi (27) in 1972. They immunized rabbits intramuscularly with injections of either egg albumin (EA) or dinitrophenyl bovine serum albumin (DNP-BSA) emulsified in complete Freund's adjuvant. Sera were collected on day 70 and analyzed for precipitating and nonprecipitating antibodies. The precipitating antibodies were removed from the antisera by serially absorbing it with antigen. The nonprecipitating antibodies remaining in the supernatant after complete absorption were specifically removed by applying the supernatants to columns of either polymerized EA or polymerized DNP-BSA, washing the columns, and subsequently eluting the antibody from them. Precipitating and nonprecipitating antibody were then tested for activity in the passive hemagglutination test and in the passive cutaneous anaphylaxis (PCA) reaction. They were both reactive, but the precipitating antibody was significantly more effective. Both types of antibody were tested for ability to produce reverse Arthus reactions and to fix complement but only the precipitating antibody accomplished those two immunological functions (in contrast to the report of Christian (5) in which nonprecipitating antibodies were said to have fixed complement). Immunoelectrophoresis showed no differences between nonprecipitating and precipitating antibody, regardless of their specificity (anti-EA or anti-DNP-BSA). No precipitin arcs were obtained in an Ouchterlony test by using the specific antigen and its homologous nonprecipitating antibody (as would be expected for an antibody that did not precipitate). The molecular size of IgG and the nonprecipitating antibodies was deemed to be similar by passing a mixture of IgG and radiolabelled nonprecipitating

antibodies through Sephadex G-200. These authors stated that their findings were compatible with the idea of nonprecipitation being due to a particular configuration of the molecule which prevents one antibody molecule from binding two different antigen molecules simultaneously.

Nonprecipitating antibodies have also been described in the dog. Patterson and his associates (30), in 1964, studied canine precipitating and nonprecipitating antibodies. They injected dogs every 2 weeks with BSA emulsified in complete Freund's adjuvant. They analyzed the sera by the quantitative isotope precipitin technique and found incomplete precipitation of the iodinated antigen in the zone of antibody excess. Iodinated antigen present in the supernatants of the precipitin test was precipitated with 50% saturated ammonium sulfate, thus showing that the iodinated antigen was in the form of soluble antigen-antibody complexes. Two of the dogs showed high hemagglutination titers but gave negative precipitation reactions. Their sera did, however, contain antibody which specifically bound antigen, as assayed by the Farr technique. The nonprecipitating antibodies showed inhibition of precipitation reaction between precipitating antibody and BSA in the zone of antibody excess following incubation of the nonprecipitating antibodies with the antigen prior to the addition of the precipitating antibody. The nonprecipitating antiserum was electrophoresed on starch block and the segments that were considered to have migrated as gamma, beta-2, and beta-1 globulins were eluted. Increasing amounts of labelled BSA were added to the eluates and the binding activity was assayed by the Farr technique. Although this type of electrophoresis did not result in a completely purified protein, the majority of the nonprecipitating antibody migrated as a beta-2 globulin, rather than a gamma globulin.

Further work on the canine nonprecipitating antibodies was reported by

Patterson, et al. (31). The immunization was done as before. They found by autoradiographic analysis that nonprecipitating serum showed a single precipitin arc, migrating faster than the slow gamma globulin, whereas the precipitating serum showed two arcs, one located as in the nonprecipitating serum and the other at the place of the slowest migrating gamma globulin. Experiments to measure the retention of immune complexes showed that when nonprecipitating serum was used after initial injection of the iodinated BSA, there was an initial retention of the antigen in the animal (retained for 24 hours in a 6-day study) followed by an elimination of the antigen from circulation. The nonprecipitating antibodies gave negative results for passively sensitizing a guinea pig for systemic or cutaneous anaphylaxis or for the Arthus reaction.

Grant and his associates (14) in 1972 reported the amino acid sequences of the 18 amino acids located on the carboxy-terminal and of the canine IgGd. This immunoglobulin subclass migrates more cathodally than other canine IgG's and does not precipitate multideterminant antigens. They found this canine immunoglobulin to be related to other IgG's but found no sharing of unique sequence between it and equine IgG(T) (a subclass of nonprecipitating antibodies in the horse).

Johnson and Renfer (16) later showed the presence of two inter-heavy disulfide bonds located between the Fd fragments of specifically purified IgGd antibodies to the DNP hapten. They proposed that these two disulfide bonds might cause a functional univalency of this subclass of immunoglobulins due to restricting the rotational freedom in the area of the binding sites of the antibody molecule when it comes into contact with large multivalent antigens.

Nonprecipitating antibodies have also been described in the horse.

Pappenheimer, in 1940 (29), described the presence of soluble antigen-antibody complexes in the serum of a horse during the early stages of immunization with recrystallized hen egg albumin. The hypothesis of univalency of the antibody molecule was thought to account for the failure of precipitation. He proposed the same idea as Heidelberger (15) that more "divalent" antibody was made later in the response to account for the precipitation seen later in the response.

Antibodies, present in the serum of a horse immunized with HSA, that would not precipitate but which would cause inhibition of precipitation by the precipitating antibody and antigen were observed by Gitlin et al. in 1949 (13). These antibodies appeared early in the course of immunization.

Some properties of a purified equine nonprecipitating antibody made against the hapten p-azophenyl-B-lactoside (Lac) were reported in 1964 by Klinman, Rockey, and Karush (23). The horse was immunized repeatedly over a period of 3 months with KLH-Lac and its serum was analyzed for specific antibodies with HSA-Lac as the antigen, to insure that only anti-hapten antibodies were being studied. The serum showed no precipitating capacity after 90 days of the immunization schedule. Purification of this serum for the specific anti-hapten antibodies was accomplished by precipitating the serum with KLH-Lac and specifically extracting the coprecipitated anti-Lac antibodies with 0.5 M lactose in 0.15 M NaCl at 37°C. The nonprecipitating antibodies were found to be divalent with a high antigen affinity, thus demonstrating that nonprecipitation was not due to low affinity or univalency.

The presence of disulfide bonds, located similarly to those of the nonprecipitating IgGd canine antibodies, in the equine nonprecipitating antibody IgG(T) seemed to suggest that a similar mechanism for failure of

precipitation might be operating in the horse (42).

The idea that the IgG(T) (a subclass of nonprecipitating antibodies in the horse) did not precipitate because the antibody found it energetically favorable to occupy both of its binding sites with two Lac haptenic groups present on the same protein molecule (HSA-Lac) was suggested by Klinman and Karush in 1967 (22). Immunization, purification, and test antigen were the same as reported earlier. Complex formation by both IgG(T) and IgG with HSA-Lac and Lac dye were studied by using immuno-adsorbent columns. The columns were made with HSA-Lac to specifically absorb the anti-Lac antibodies from the purified preparations and the ability of the antibody to bind two separate antigen molecules was measured by pouring a solution of labelled HSA-S-Lac through the column. Alternatively, the anti-Lac antibodies were incubated with ^3H -Lac dye, the HSA-Lac immuno-adsorbent was added, and tritium uptake onto the immuno-adsorbent column was measured. The results led to the conclusion given at the beginning, i.e., that this antibody showed monogamous bivalency. That this was a plausible explanation was substantiated by electron microscopic studies by Feinstein and Rowe (11). They used ferritin and rabbit IgG anti-ferritin and found suggestive evidence for a 1:1 ratio of antigen to antibody in the zone of antigen excess.

Sera from man has also been the source of nonprecipitating antibody. Although many various studies have been done, just one example is given here. Skom and Talmage (38) in 1958 demonstrated the presence of nonprecipitating antibodies made against insulin in the serum of a diabetic patient receiving insulin injections. These antibodies were detected using the anti-globulin technique for detection of primary interaction of antigen and antibody.

In the past most researchers in the area of nonprecipitating antibodies used heterologous antigens, such as BSA. This was an erroneous approach because nonprecipitability with heterologous antigens may be due to the structure of the antigen and not due to some unique characteristic of the antibody which will not allow it to precipitate. In this study, therefore, an antigen system was used in which a single determinant site, the hapten, was multiply coupled to the carrier protein. This type of antigen was precipitable because it contained several of the same antigenic sites to which specific antibodies could bind and form the lattice structure necessary for precipitation. The only reason for any observed nonprecipitability in this system would be a function of the specific antibodies and not of the antigen.

The combination of the Farr technique, radial immunodiffusion, and calculation of molar ratios of antigen bound:precipitating antibody was used as a new approach to assaying for the presence of nonprecipitating antibodies.

MATERIALS AND METHODS

I. Buffers and Reagents*

A. Borate buffer.

Borate buffer was made from 156g NaCl + 200g boric acid + 20g NaOH + deionized water to a final volume of 20 liters.

Final pH was 8.0 without adjustment.

Borate saline buffer (BSB) was made from 1 part borate buffer : 9 parts physiological saline.

B. Glycine buffer.

Glycine buffer (0.2 M) was made from 15g glycine (Mallinckrodt Chemical Works, St. Louis, Mo.) + deionized water to a final volume of 1 liter. The pH was adjusted to 8.6 with NaOH.

C. Lactate barbital buffer.

The chemicals used to make this buffer were all obtained from Fisher Scientific Company, Fairlawn, N.J.

Lactate barbital buffer was made from 8.76g sodium barbital + 1.38g diethyl barbituric acid + 0.384g calcium lactate + deionized water to a final volume of 1 liter. The pH was adjusted to 8.6 with NaOH.

D. Sodium bicarbonate buffer.

Sodium bicarbonate buffer (0.1 M) was made from 8.4g sodium bicarbonate (Matheson, Coleman, and Bell Manufacturing Chemists, Norwood, Ohio), + deionized water to a final volume of 1 liter. The pH was adjusted to 9.0 with NaOH.

E. Tris-(hydroxymethyl)-amino methane (Tris)-HCl buffer.

*Chemicals used were of reagent grade.

Tris buffer was made from 12.1g of Tris (Fisher Scientific Company, Fairlawn, N.J.) + deionized water to a final volume of 1 liter. The pH of this 0.1 M solution was adjusted to 7.6 with HCl.

F. Tris-HCl buffer containing 8 M urea.

This Tris-HCl buffer was made from 12.1g Tris + 480g urea (Difco Laboratories, Detroit, Michigan) + deionized water to a final volume of 1 liter. The pH was adjusted to 7.6 with HCl.

G. Cyanogen bromide (BrCN) was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin.

H. Iodine monochloride (ICl) was obtained from Eastman Kodak Company, Rochester, New York.

I. Polyvinylacetate-polyvinylchloride copolymer (Pevikon) was obtained from Mercer Chemical Corporation, New York, New York.

J. Propionic acid, reagent grade, was redistilled prior to use.

K. Radioactive iodine (^{125}I) was obtained from New England Nuclear, Des Plaines, Illinois. The molecular form was NaI and the specific activity was 17 Curies/mg (carrier free, greater than 99% pure).

L. Saturated ammonium sulfate (SAS) was made by adding ammonium sulfate to about 4 liters of deionized water, mixing, and adding more ammonium sulfate until a saturated solution was made. The final pH was 7.0 without adjustment.

M. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

N. Stain used for radial immunodiffusion slides was made by dissolving 1g of Light Green S F Yellowish stain in a final

volume of 200 ml of a solution made of 7 parts methanol : 2 parts water : 1 part glacial acetic acid. This solution (without stain) also served as the 3 rinses used after the stain.

- O. Trichloroacetic acid (20%) was made by dissolving 20g of trichloroacetic acid (Mallinckrodt Chemical Works, St. Louis, Mo.) in a total volume of 100 ml deionized water.
- P. "Ionagar" #2 (Ionagar) was obtained from Colab Laboratories, Incorporated, Chicago Heights, Illinois.

II. Hapten and Carrier Proteins

A. Hapten.

The hapten used was p-aminophenyl-N-trimethyl ammonium chloride (R_4N). It was synthesized and recrystallized from methanol by Dr. V. Peter Kreiter, Red House Laboratory, Prantham Road, Glenwood, N.Y. 14069. This was used for the hapten for both the immunizing antigen and the testing antigen.

B. Keyhole limpet hemocyanin (KLH).

Crystallized hemocyanin from the giant keyhole limpet was obtained from Mann Research Laboratory, Becton-Dickinson and Company, Rutherford, N.J. It was used as the carrier protein portion of the immunizing antigen.

C. Bovine serum albumin (BSA).

Crystallized bovine albumin was obtained from Pentex Biochemicals, Kankakee, Illinois. It was used as the carrier protein portion of the testing antigen.

III. Diazotization Procedure

The diazotization procedure (43) was used to prepare the test antigen (BSA-R⁴N) and the immunizing antigen (KLH-R⁴N). Two forms of test antigen were made, one called highly coupled and the other called less highly coupled. The only difference between the two was the way they were made. The highly coupled test antigen was made to be used in the radial immunodiffusion test and for the immunoadsorbent. The less highly coupled test antigen was made to be used in the Farr technique and in the quantitative precipitin test.

A. Preparation of less highly coupled test antigen.

One gram of BSA was dissolved in 50 ml of BSB. The pH was adjusted to 9.5 with NaOH just prior to diazotization. Forty (40) mg of R⁴N were dissolved in 5 ml of 1 M HCl and kept in an ice bath. A 1% solution of NaNO₂ in water was added dropwise until a drop of the mixture gave the first hint of blue color on starch-KI paper. The titrated hapten was added to the rapidly stirring protein while maintaining the pH at 9.0-9.5 with 1 M NaOH. The resulting test antigen was dialyzed exhaustively against BSB at 4°C (dialyzed about 1 week with changes of BSB twice daily).

B. Preparation of highly coupled test antigen.

This test antigen was prepared as above except that one-half the amount of carrier protein (BSA) was used with the same amount of hapten.

C. Preparation of immunizing antigen.

This antigen was prepared as the less highly coupled test antigen except that KLH was used in place of BSA for the carrier protein.

IV. Antiserum Production

A. Method of immunizing and collecting blood.

Each New Zealand White rabbit was immunized subcutaneously in the two diagonal quadrants of its back with 2 ml containing 10 mg of KLH-R⁴N emulsified in a 1:1 ratio of antigen : complete Freund's adjuvant (H37Ra, Difco Laboratories, Detroit, Michigan). Blood was collected from the rabbits by nicking the marginal ear vein after the area had been washed with alcohol and the vein dilated with xylol.

B. Bleeding and immunization schedule.

Each rabbit was bled just prior to the initial injection and every 3 weeks thereafter for the duration of the schedule. The rabbits were actually bled on the 2 days on each side of the specified day. The sera were pooled from each of these two days and labelled as the specified day. For example, the rabbits were bled on days 20 and 22, the sera were pooled and labelled day 21.

Sera were collected on days 21, 42, 63, 84, 149, 171, 190, 212, and 251 for the "B" series of rabbits. Sera were collected on days 21, 42, 63, 84, 104, 119, 126, 132, and 146 for the "C" rabbits (except for days 132 and 146 for rabbit C38). This second series of rabbits were bled on two days (119 and 132) that were not 3 weeks after immunization. This was done in an effort to follow more closely their nonprecipitating antibody response.

Each rabbit was immunized as previously described, every 3 weeks for the duration of the schedule. The actual injection took

place after bleeding the rabbits, on the second of the 2 days that they were bled. For example, the second bleeding for the first three weeks was on day 22. On that day the rabbits were injected, after they had been bled. The immunization schedule lasted 212 days for the "B" rabbits and 146 days for the "C" rabbits (discontinued at that time because the molar ratios leveled off).

V. Radiolabelling

The procedure used for radiiodination was a slight modification of the technique of McFarlane (25).

A. Preparation of antigen.

Less highly coupled BSA-R⁴N was diluted with glycine buffer to a final volume of 1 ml and dialyzed against glycine buffer overnight at 4°C.

B. Quantitation of ¹²⁵I.

Prior to iodination the stock solution of ¹²⁵I was quantitated by adding 1 drop of it to 100 ml of deionized water, mixing well, and counting duplicate 1 ml aliquots in a Nuclear-Chicago gamma counter. Counts per minute (cpm) contained in that original 1 drop were calculated by multiplying the cpm (obtained from the gamma counter) by 100 because only 1 ml aliquots of the original 100 ml were counted. The desired amount of labelling was $1.0 \times 10^3 - 4.0 \times 10^3$ cpm per ug protein and the labelling efficiency was considered to be 50%. Therefore, the ug of protein to be labelled were multiplied by 2.0×10^3 and 8.0×10^3 cpm to give the range of desired cpm. The number

of drops of ^{125}I necessary to give the desired amount of label were calculated by dividing the desired cpm by the cpm per drop. The same Pasteur pipette that had been used to deliver the 1 drop for quantitation purposes was used to add the ^{125}I to the glycine buffer during the iodination procedure.

C. Quantitation of ICl.

The ICl solution used in the procedure contained 1 mg/ml of ICl and was prepared from redistilled ICl by adding 0.1 ml of the ICl to 330 ml of 0.1 M HCl. The following calculation was done to determine the amount of ICl necessary for iodination:

$$\text{ICl(mg)} = \frac{\text{mg protein to be labelled} \times \# \text{ atoms/molecule labelled} \times 163}{\text{M.W. of protein to be labelled}}$$

In this study 1.5 atoms/molecule was used as the desired amount of label. The M.W. of BSA was 67,000 and the M.W. of the ICl was 163. This calculation resulted in mg of ICl necessary to label the protein with 1.5 atoms ^{125}I /molecule. The mg of ICl was easily converted to ml of ICl since a concentration of 1 mg/ml ICl was used.

D. Iodination procedure.

The ^{125}I and ICl were added to 0.4 ml of the glycine buffer, mixed, and added dropwise to a vial containing 0.5 ml of the antigen-glycine buffer mixture. The resultant solution was stirred and dialyzed for 1 hour against BSB containing a final concentration of 0.005 M KI. The iodinated solution was then dialyzed against BSB at 4°C for at least 12 hours and the BSB was assayed for radioactivity by counting duplicate 1 ml

aliquots of it in the gamma counter. This last step was done to determine that dialysis had gone to completion.

E. Assay of labelling efficiency.

Trichloroacetic acid (TCA) precipitation was used to assay for the labelling efficiency of the iodination procedure. The Pasteur pipette used to transfer the iodinated antigen to the dialysis bag was rinsed into a tube containing 1 ml of a 1 mg/ml solution of human gamma globulin (HGG) (obtained from Pentex Biochemicals, Kankakee, Ill.) as the carrier protein. One ml of 20% TCA was added, the tube was immediately vortexed, and allowed to stand at room temperature (T.T.) for approximately 1 hour. After incubation the precipitate was collected by centrifuging in an International refrigerated centrifuge, model PR-6 (IEC) at 2,000 rpm (1,000xg) for 15 minutes. The supernatant was saved for later assay. Two ml of 10% TCA (10g of TCA + deionized water to a final volume of 100 ml) was added to the precipitate, the tube was vortexed, and centrifuged as before. This procedure was repeated, resulting in 3 separate supernatants. Following centrifugation, the precipitate was dissolved in 1 M NaOH in a final volume of 2 ml. Count rates of these 4 tubes were determined in the gamma counter. The efficiency of labelling was calculated by multiplying the percentage of the total cpm present in the precipitate by the desired number of atoms per molecule.

VI. Antibody Assay

A. Quantitative precipitin test.

The quantitative precipitin test (15) was used as a quantitative measure for precipitating antibodies.

A constant volume of serum (either 0.5 or 1.0 ml) plus a variable quantity of BSA-R₄N (less highly coupled) contained in a constant volume (0.5 or 1.0 ml) were mixed together in 12 x 75 mm tubes, incubated for approximately 3.5 hours at 37°C followed by approximately 36 hours at 4°C. The precipitates were then collected by centrifuging at 2,500 rpm (1,500 x g) for 15 minutes and the supernatants were discarded. The precipitates were washed and recollected twice, discarding the supernatants each time. The precipitates were dissolved in 1 ml of 0.05 M NaOH and the resultant solutions were read, using a Gilford Spectrophotometer, at 280 and 365 nm (the latter wavelength being that giving maximum absorbance for the antigen). Two sets of graphs were constructed; one represented a rough estimate of the zone of equivalence and the second gave a finer determination of the equivalence zone, by using smaller increments of increase of antigen quantity (hence showing the maximum amount of precipitate formed). An "F" value was calculated for each preparation of BSA-R₄N used in the quantitative precipitin test. The "F" value was used as a correction factor to subtract the protein absorbance of the antigen from the total absorbance read on the spectrophotometer for each tube of dissolved antigen-antibody precipitate. The calculation was done as shown in the following equation:

$$"F" = \frac{O.D._{280 \text{ nm}}}{O.D._{365 \text{ nm}}}$$

where $O.D._{280 \text{ nm}}$ = absorbance due to the BSA portion of the antigen

$O.D._{365 \text{ nm}}$ = absorbance due to the diazo bond between the BSA and R4N.

The calculation for the precipitating antibody concentration was done using the following equation:

$$O.D._{280 \text{ nm}} = \text{Total } O.D._{280 \text{ nm}} - (O.D._{365 \text{ nm}} \times "F")$$

(where total $O.D._{280 \text{ nm}}$ is that which is read directly with the spectrophotometer, $O.D._{365 \text{ nm}}$ is that which is read with the spectrophotometer using the same dissolved precipitate as used for the total $O.D._{280 \text{ nm}}$, and $O.D._{280 \text{ nm}}$ is the absorbance at 280 nm due to just the antibody present in the dissolved precipitate). The calculation for the concentration of precipitating antibody was done using the above $O.D._{280 \text{ nm}}$ value. The concentration was calculated by dividing the $O.D._{280 \text{ nm}}$ value by 1.5 to give the concentration in mg/ml because the extinction coefficient for a 1% solution of rabbit gamma globulin read at 280 nm in a cuvette with an inside diameter of 1 cm is 15 (44).

B. Farr technique (ABC test).

The ABC test (9) was used to measure primary antigen binding capacity. Its underlying principle is that antigen-antibody complexes, soluble in antigen excess, are precipitable by 50% SAS. The precipitate contains antigen-antibody complexes and serum globulins (globulins, but not albumin are also precipitated by the salt). The antigen used in this test is radiolabelled so that it can be detected either in the supernatant (unbound) or in

the precipitate (bound).

Normal rabbit serum (NRS) was collected from healthy unimmunized rabbits and was used to make a diluent to dilute antigen and samples in the test. The diluent was made by mixing 9 parts BSB : 1 part NRS (previously inactivated for complement by heating at 56°C for 30 minutes).

Two types of controls were used for each ABC test. A nonspecific binding control (NSB Ct) was made by adding 0.5 ml of the diluent and 0.5 ml of the antigen and thereafter treated as one of the serum samples. This control was used to show how much of the antigen binding results were due to the diluent used. A trichloroacetic acid control (TCA Ct) was made the same way as the NSB Ct but, after the initial incubation at 4°C, 1 ml of 20% TCA was added. This latter control was then treated as all the other samples except that the precipitate was initially dissolved by 1 M NaOH. The values obtained in this control were used to show the maximum amount of iodinated antigen that could be precipitated or the maximum cpm that would result if all the iodinated antigen were bound and hence precipitated with 50% SAS.

The antigen used was iodinated BSA-R₄N (less highly coupled) at a concentration of 2 ug per ml. Each tube, including the controls, received 0.5 ml (1 ug) of the antigen. Each serum or purified antibody preparation that was assayed by the ABC test was diluted with the diluent already described, usually starting with a dilution of 1:10 and making 5-fold dilutions thereafter as far as

necessary to get a binding percentage less than the ABC-33% endpoint. One-half (0.5) ml of each dilution was added to each of two duplicate tubes and the controls were prepared in duplicate, as previously described. Following the addition of the iodinated antigen to each tube, the samples were vortexed, incubated for at least 1 hour at R.T., and then incubated at 4°C overnight. The TCA Ct tubes received 1 ml of 20% TCA apiece but all the other tubes received 1 ml of SAS apiece. The tubes were immediately vortexed at 4°C and left at 4°C for at least 30 minutes. The precipitates were then collected by centrifugation at 2,000 rpm (1,000 x g) for 20 minutes, the supernatants discarded, and all the precipitate (except the TCA Ct precipitate which was dissolved in 1 ml of 1 M NaOH) were dissolved with 1 ml of 0.05 M NaOH. These dissolved precipitates were transferred to separate counting tubes. Another 1 ml of 0.05 M NaOH was added to the test tubes, vortexed, and the solution transferred to its respective counting tube. All of the counting tubes plus two blank counting tubes (to show background cpm) were counted in the gamma counter.

The following calculations were done to determine antigen binding capacity of each sample. The radioactivity present in each tube was corrected for background cpm. The corrected cpm for the NSB Ct and TCA Ct were averaged, separately. The average for the NSB Ct was subtracted from the corrected cpm of each tube. The resultant cpm of each tube was then divided by the averaged TCA Ct value to give the percentage of iodinated antigen present

in each precipitate. The percentages of each pair of tubes were averaged. The dilutions of each sample tested and its corresponding percentage of iodinated antigen precipitated were graphed on semilogarithmic paper, with the dilutions plotted on the logarithmic axis and the percentages on the arithmetic axis. The values above and below the 33% endpoint were used to draw the intersecting line through the 33% value (see Figure I). The reciprocal of the dilution necessary to give 33% of the iodinated antigen bound (also known as the ABC-33 endpoint) was used in the following equation to calculate the binding values per ml of undiluted sample:

$$\text{ug } ^{125}\text{I-BSA-R4N bound/ml} = \text{ABC-33 endpoint} \times 2 \times 0.33 \times 1 \text{ ug antigen}$$

ABC values (ug iodinated antigen bound/ml) were expressed as ug antigen bound/ug protein for the purified samples because their protein concentrations per ml were known.

C. Radial immunodiffusion (RID).

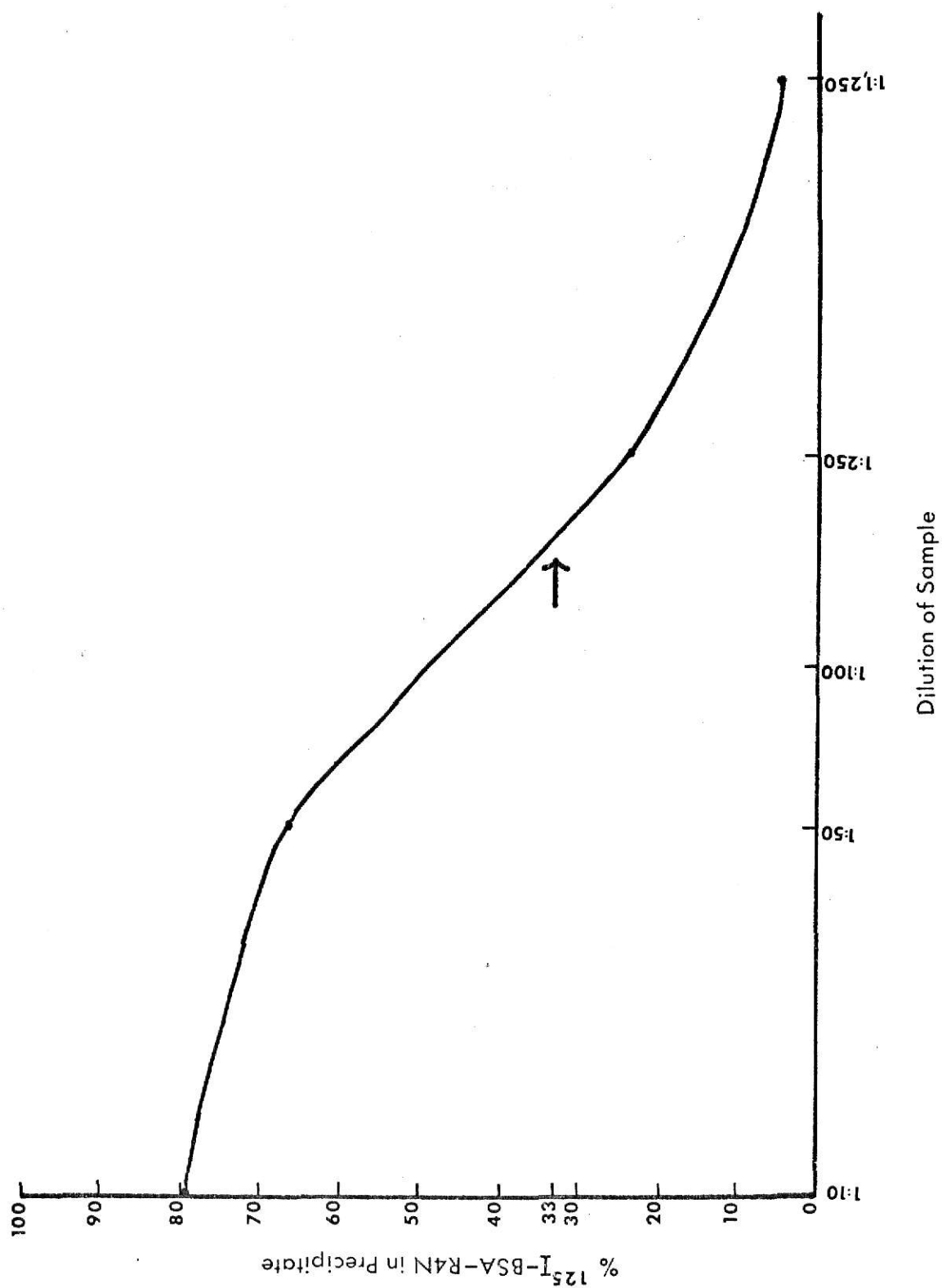
This method (26) was used to determine the concentrations of precipitating antibody present in both sera and in purified antibody samples. The RID test was standardized by using various dilutions of sera previously analyzed by the quantitative precipitin technique.

Agar was made using Ionagar with BSB and approximately 0.002% sodium azide (to inhibit bacterial growth) and was autoclaved for 20 minutes at 15 psi. Samples to be analyzed were inactivated for complement (56°C for 30 minutes), although some preliminary

FIGURE I. Graph showing an example of the method of plotting ABC data. This curve for B26-212 indicates a 33% endpoint dilution value of 1:172. The ABC value is 114 ug antigen bound/ml of undiluted serum.

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data indicated that this step was not required. A total of 3 ml of sample-agar mixture was layered onto a microscope slide, previously coated with approximately 0.5% Ionagar made with BSB. The final concentration of the agar was 1% (agar of various concentration was made, depending on the amount of sample to be mixed with the agar). After the agar-sample mixture had solidified, a series of 2.0 mm wells were punched out of each slide (usually 3 pairs of wells). Duplicate wells were filled with antigen concentrations of 250 ug/ml, 500 ug/ml, and 1,000 ug/ml, respectively. The slides were incubated in a humid chamber for 25 hours at R.T. and then washed with BSB in a continuous flow bath, using usually 4 or 8 liters, depending on how many slides were being washed. The slides were then dried, stained with 0.5% Light Green S F Yellowish for 15 minutes, and rinsed three times for 15 minutes apiece in a solution described earlier. The stained rings on the slides, including control rings, were then measured using a photographic enlarger and the actual diameters were read from a graph prepared by measuring several actual distances by a vernier caliper and plotting actual distances versus enlarged diameters as read using the enlarger.*

The concentration of precipitating antibody was determined from two graphs, one using the 250 ug/ml antigen concentration and the other using the 500 ug/ml concentration. Both graphs were

*The use of two antigen concentrations, as well as doing duplicates, provided controls for the system.

made with various concentrations of precipitating antibody by using dilutions of sera analyzed by quantitative precipitin test. Actual ring diameter versus amount of precipitating antibody/slide was plotted for each graph. The concentration of precipitating antibody was determined per ml of sample since the volume of sample put onto the slide was known. The concentration per ug protein was calculated for purified antibody samples.

VII. Molar Ratio Calculations

A. Example for a low molar ratio serum (820-84).

ABC value = 132 ug antigen (Ag) bound/ml of serum

RID value = 229 ug precipitating antibody (Ab)/ml of serum

$$\text{Moles of Ag bound/ml} = 132 \text{ ug Ag} \times \frac{1 \text{ mole Ag}}{6.7 \times 10^{10} \text{ ug}} = 1.97 \times 10^{-9} \text{ moles Ag}$$

$$\text{Moles of Ab/ml} = 229 \text{ ug Ab/ml} \times \frac{1 \text{ mole Ab}}{1.5 \times 10^{11} \text{ ug}} = 1.53 \times 10^{-9} \text{ moles Ab}$$

Molar ratio of moles Ag bound/ml : moles Ab/ml =

$$\frac{1.97 \times 10^{-9}}{1.53 \times 10^{-9}} = 1.29 : 1.00$$

B. Example for a high molar ratio serum (B21-190).

ABC value = 92.5 ug Ag bound/ml of serum

RID value = 68 ug Ab/ml of serum

$$\text{Moles of Ag bound/ml} = 92.5 \text{ ug Ag} \times \frac{1 \text{ mole Ag}}{6.7 \times 10^{10} \text{ ug}} = 1.38 \times 10^{-9} \text{ moles Ag}$$

$$\text{Moles of Ab/ml} = 68 \text{ ug Ab/ml} \times \frac{1 \text{ mole Ab}}{1.5 \times 10^{11} \text{ ug}} = 4.53 \times 10^{-10} \text{ moles Ab}$$

Molar ratio of moles Ag bound/ml : moles Ab/ml =

$$\frac{1.38 \times 10^{-9}}{4.53 \times 10^{-10}} = 3.05 : 1.00$$

VIII. Specific Immunochemical Method of Antibody Purification

Antibodies specific for the R⁴N hapten were purified using a solid phase immunoabsorbent. Briefly, the procedure involved the covalent attachment of hapten-carrier to a solid matrix of Sepharose 4B (1, 8, and 33).

A. Preparation of immunoabsorbent.

Two guidelines that were used in the procedure were 1) 2 mg of protein to be attached : 1 g of Sepharose and 2) 1 g of Sepharose : 1 ml of sodium bicarbonate buffer.

The procedure given was used regardless of amount of Sepharose 4B (Sepharose) to be activated. The quantities given are those used for activating 125 g of Sepharose.

Preparatory steps to be done the day before activation included making 4 liters of sodium bicarbonate buffer to be stored overnight at 4°C, along with 1 liter of deionized water. A 125 g quantity of Sepharose was weighed out and suspended in 125 ml of the sodium bicarbonate buffer. The pH was then adjusted to 9.0 with NaOH and the slurry was stored at 4°C overnight. A 250 g quantity of highly coupled BSA-R⁴N was dialyzed against 1 liter of sodium bicarbonate buffer overnight at 4°C. The next

day 16.7 g (\pm 0.2 g) of BrCN was dissolved in 125 ml of deionized water plus 20 ml of dimethylformamide (the latter to make the BrCN go into solution more quickly). The Sepharose was stirred in an ice bath under a pH meter and pH was quickly adjusted to 11.0-11.5 with 4 M NaOH. The BrCN solution was poured in and the pH was maintained at 11.0-11.5 with 4 M NaOH for 9 minutes. The slurry was then washed under suction first with cold deionized water followed by cold sodium bicarbonate buffer. The Sepharose was resuspended to its original volume with sodium bicarbonate buffer. The elapsed time for washing should be as short as possible, preferable under 2 minutes. The Sepharose slurry was then stirred and the protein was added dropwise while the pH was maintained at 9.0-9.5. The pH was checked for an hour to be sure that it was stabilized. The slurry was then stirred overnight at 4°C. The next day the slurry was put into a column and washed with approximately 1.700 ml of 0.01 M NH_4OH , pH 9.0. The slurry was then resuspended in BSB and stored at 4°C until used.

B. Immunosorption and elution.

The guideline used for both methods was to use a ratio of 1:2 of column : serum volume.

1. Method using BSB to buffer and propionic acid to elute.

Each column was packed with the Sepharose-BSA- $\text{R}^{4\text{N}}$ and was washed with a volume of BSB approximately equal to 6 times the column volume. The serum was then applied to each column and allowed to run through slowly. After the meniscus of the serum reached the top of the column,

enough BSB was run through the column to get a final effluent volume of 10 times that of the serum applied to the column (to give a 1:10 dilution to assay by the ABC test to be sure that all the anti-hapten antibody was absorbed by the column). Propionic acid (1 M, pH 2.6) was run through the column and the elution of antibodies was monitored by reading the O.D._{280 nm} on the spectrophotometer. The elution was discontinued after the absorbance dropped below 0.100 O.D. unit. The purified antibody solution was then neutralized to pH 7.0 with NaOH and dialyzed against BSB overnight.

2. Method using Tris-HCl buffer and Tris-HCl buffer containing 8 M urea.

The columns were washed with 0.1 M Tris-HCl buffer, pH 7.6, before the serum was applied. The final effluent volume was ten times that of the serum sample applied, as in the previously described method, except that Tris-HCl buffer was used instead of BSB. The elution was then accomplished with 0.1 M Tris-HCl buffer containing 8 M urea, pH 7.6, and was monitored by the spectrophotometer as described previously. The purified preparations were then dialyzed against BSB overnight at 4°C.

IX. Physicochemical Characterization of Antibodies

A. Preparative electrophoresis.

This procedure was used to separate the components of the purified antibody preparations on the basis of differing

migration rates during electrophoresis. The electrophoresis unit used was a Model 800 obtained from Scientific Manufacturing Industries.

Approximately 800 ml of dry Pevikon was brought to a total volume of 2 liters with deionized water and washed by stirring and allowing it to settle. The supernatant was decanted and the Pevikon was then washed with 2 liters of 0.1 M NaOH, followed by washing with deionized water until the pH stabilized (approximately pH 5.2). One liter of lactate barbital buffer was then added to the washed Pevikon. The electrophoresis bed was sealed and a slurry of approximately 1 part lactate barbital buffer : 3 parts Pevikon was poured into the bed. The slurry was smoothed out and dried with paper toweling until the slurry just lost the wet sheen. A trough to hold the sample was cut 5 inches from the cathode end and $5\frac{1}{4}$ inches across the width of the bed (centrally located with $1\frac{1}{2}$ inches on either side to the edge) by $\frac{3}{8}$ inch across the length of the bed. For a 3 ml sample the dimensions were the same except that the trough was 2 inches across the width of the bed. The sample was electrophoresed for 18 hours, while the voltage across the bed was maintained at 200 volts (6 volts/cm). Prior to electrophoresis the reservoirs were filled with lactate barbital buffer and Whatman chromatography paper was used for wicks to connect reservoirs and bed.

After electrophoresis, the Pevikon was cut into approximately 33 narrow strips, from the cathode end to the anode end. For

a 5 ml sample the strips were 21 cm by 1 cm and for a 3 ml sample they were 10.2 cm by 1 cm. These strips were removed from the bed and the protein was eluted with lactate barbitol buffer (5 ml for a 5 ml sample and 3 ml for a 3 ml sample). They were mixed well, the Pevikon allowed to settle, and the supernatants were removed with Pasteur pipettes. The supernatants were then centrifuged at 2,000 rpm (1,000xg) for 15 minutes to remove the remaining Pevikon. The O.D._{280 nm} for each tube was read in the spectrophotometer and those tubes showing some protein were subsequently used in ABC tests.

RESULTS

The first series of rabbits used in this study, the "B" series, was immunized every three weeks for a total of 212 days. They were injected with 2 ml of an emulsion made with a 1:1 ratio of KLH-R4N : complete Freund's adjuvant, containing a total of 10 ml of KLH-R4N. The injections were given subcutaneously in the back, 1 ml into each of two diagonal quadrants. The rabbits were bled from the marginal ear vein just prior to the initial immunization and every three weeks thereafter.

The sera used for analysis were from rabbits B18, B20, B21, B22, B23, B24, B26, B27, and B28 on days 42, 63, 84, 149, 171, 190, 212, and 251. All sera were assayed for antigen binding capacity per ml of undiluted serum using the ABC test. The RID test was used to assay all sera (except days 42, 149, and 251 and day 212 for rabbit B24) for concentration of precipitating antibody per ml of serum. The quantitative precipitin test was used to evaluate sera from days 42, 149, and 251 for precipitating antibody per ml of serum.

The results of the ABC and RID tests for the "B" series of rabbits are contained in Tables I. II. and III. These results show that in some of the rabbits, B18, B20, B22, and B24, there was a concurrent increase and decrease in binding capacity and precipitating antibody concentration, resulting in 1 or 2 peaks. Rabbit B27 appeared to have 3 of these peaks. However, B26 showed a slight increase in both values and seemed to level off to a plateau value for both.

Molar ratios (moles of antigen bound/ml : moles of precipitating antibody/ml) were calculated for all these sera by using the results of ABC and RID tests. Graphs showing these results are contained in Figures II-IV.

TABLE I. ABC (ug antigen bound/ml) and RID (ug precipitating antibody/ml) values for rabbits B18, B20, and B21, bled on days 42, 63, 84, 149, 171, 190, 212, and 251.

Serum	ug Antigen Bound/ml	ug Precipitating Antibody/ml
B18-42	139	367
B18-63	158	300
B18-84	129	192
B18-149	121	166
B18-171	324	277
B18-190	416	387
B18-212	281	277
B18-251	90	423
B20-42	41	45
B20-63	117	166
B20-84	132	229
B20-149	140	293
B20-171	403	365
B20-190	214	257
B20-212	125	146
B20-251	218	385
B21-42	165	380
B21-63	212	302
B21-84	125	154
B21-149	72	100
B21-171	79	75
B21-212	83	65
B21-251	97	189

TABLE II. ABC (ug antigen bound/ml) and RID (ug precipitating antibody/ml) values for rabbits B22, B23, and B24, bled on days 42, 63, 84, 149, 171, 190, 212, and 251.

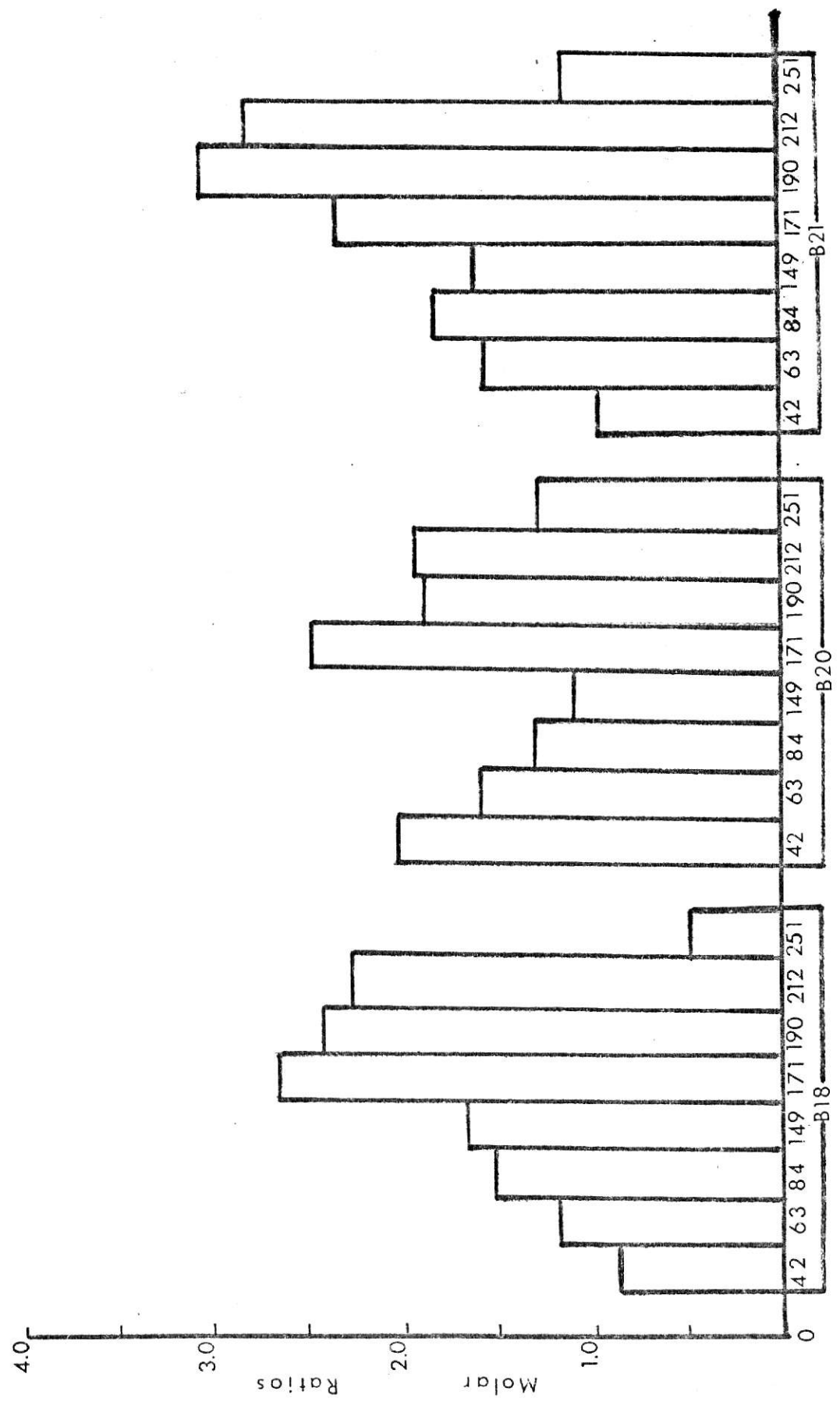
Serum	ug Antigen Bound/ml	ug Precipitating Antibody/ml
B22-42	363	713
B22-63	409	580
B22-84	595	570
B22-149	327	440
B22-171	443	453
B22-190	528	453
B22-212	448	490
B22-251	515	893
B23-42	310	893
B23-63	337	400
B23-84	228	340
B23-149	99	190
B23-171	162	107
B23-190	114	120
B23-212	104	108
B23-251	274	440
B24-42	132	275
B24-63	261	396
B24-84	290	450
B24-149	254	307
B24-171	221	312
B24-190	241	253
B24-212	168	Undetectable
B24-251	442	640

TABLE III. ABC (ug antigen bound/ml) and RID (ug precipitating antibody per ml) values for rabbits B26, B27, and B28, bled on days 42, 63, 84, 149, 171, 190, 212, and 251.

Serum	ug Antigen Bound/ml	ug Precipitating Antibody/ml
B26-42	85	157
B26-63	98	260
B26-84	110	298
B26-149	149	275
B26-171	129	264
B26-190	149	250
B26-212	114	104
B26-251	178	245
B27-42	81	165
B27-63	166	202
B27-84	137	100
B27-149	76	48
B27-171	107	289
B27-190	89	82
B27-212	102	81
B27-251	168	342
B28-42	30	135
B28-63	64	70
B28-84	63	64
B28-149	28	153
B28-171	42	45
B28-190	52	48
B28-212	42	36
B28-251	76	38

FIGURE II. Molar ratios of moles of antigen bound : moles of precipitating antibody per ml of serum for rabbits B18, B20, and B21, bled on days 42, 63, 84, 149, 171, 190, 212, and 251.

Graph of Molar Ratios of Antigen Bound : Precipitating Antibody per ml of Sera from Rabbits B18, B20, and B21



Rabbits and Days of Bleeding

FIGURE III. Molar ratios of moles of antigen bound : moles of precipitating antibody per ml of serum for rabbits B22, B23, and B24, bled on days 42, 63, 84, 149, 171, 190, 212, and 251.

Graph of Molar Ratios of Antigen Bound: Precipitating Antibody per ml of Sera from Rabbits B22, B23, and B24

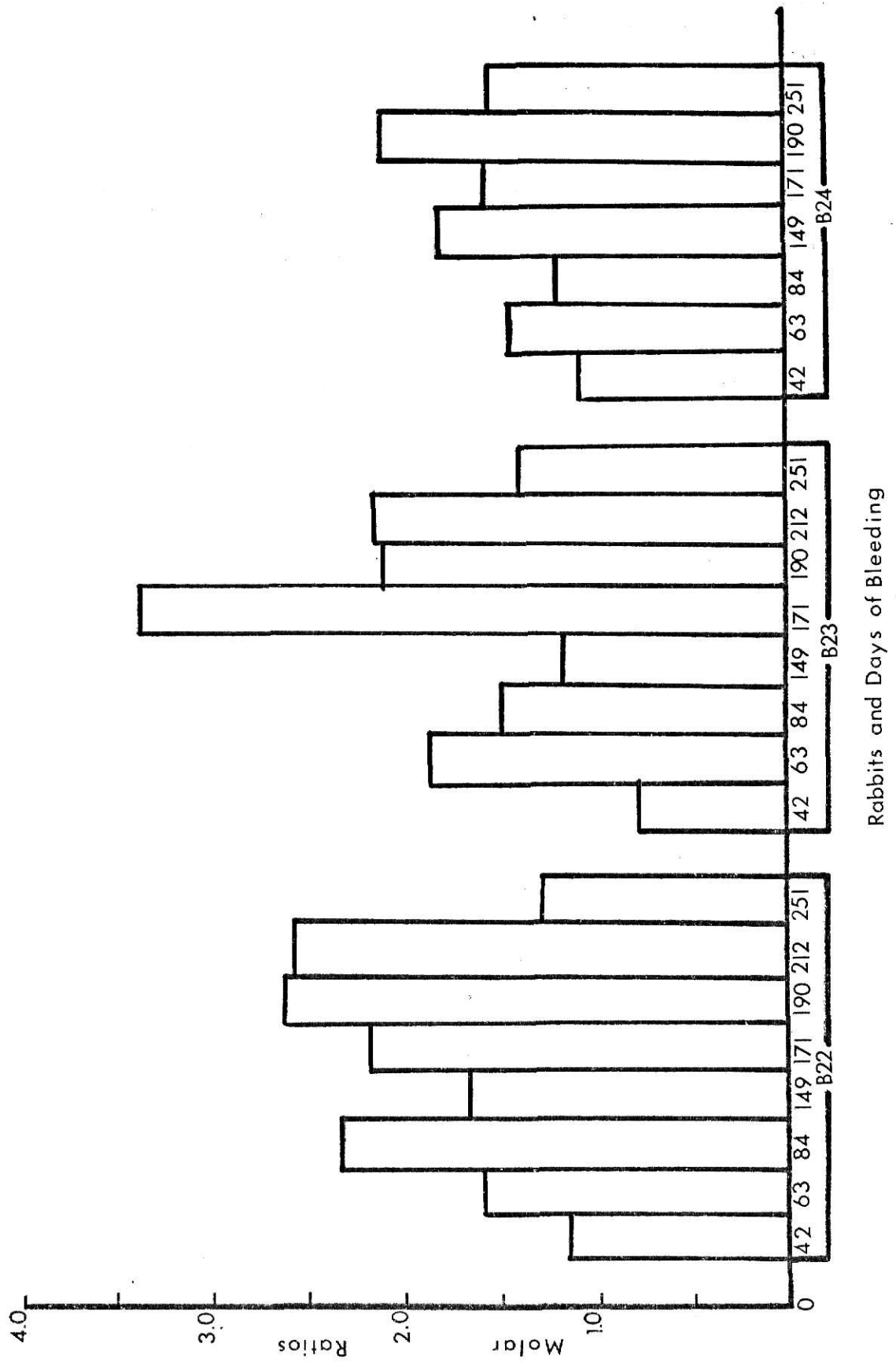
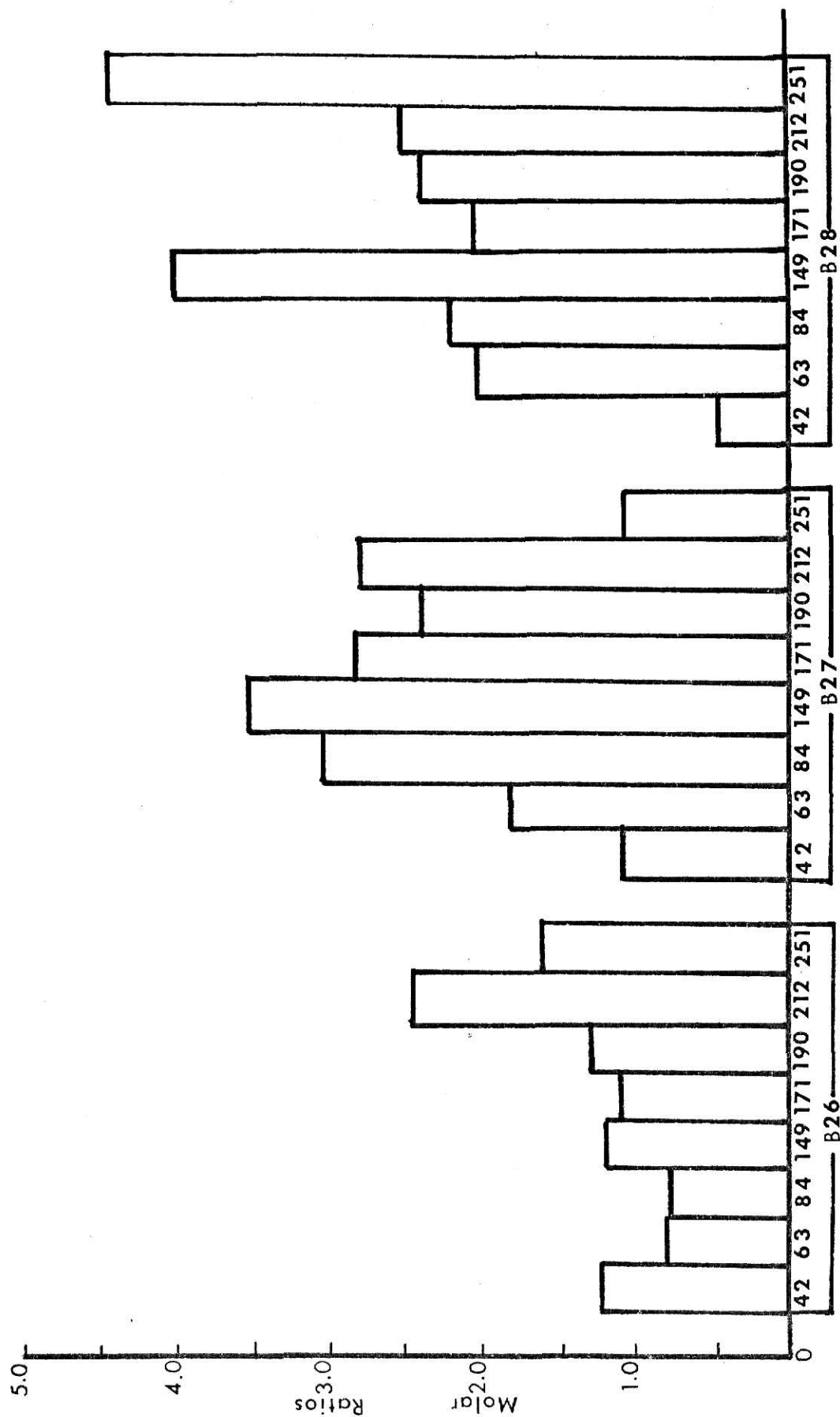


FIGURE IV. Molar ratios of moles of antigen bound : moles of precipitating antibody per ml of serum for rabbits B26, B27, and B28, bled on days 42, 63, 84, 149, 171, 190, 212, and 251.

Graph of Molar Ratios of Antigen Bound: Precipitating Antibody per ml of Sera from Rabbits B26, B27, and B28



Rabbits and Days of Bleeding

All but one of the rabbits showed an initial increase in molar ratio subsequently followed by a decline, suggesting that the molar ratio was not an invariant characteristic of the response using this immunization program. Rabbits B18, B21, B24, B26, and B27 appeared to respond with an increased molar ratio in a manner that gave a single peak. However, rabbits B22, B23, and B28 gave an increased molar response that occurred twice. This could conceivably be due to a diphasic type of antibody response, caused by two clones of antibody-producing cells synthesizing antibody, each at a different distinct time. In contrast to this, the first observation could be due to a single clone of antibody-producing cells. It is not certain what the explanation is for the results for rabbit B20 since this rabbit had an initially high molar ratio which decreased and then showed a single peak later. Later bleedings than those used in this study might have suggested something about the kinetics of the response that was not observed using the available serum samples. The later bleedings might have shown that there was a decline in the amount of nonprecipitating antibody or that this amount reached a certain concentration and remained at that concentration for a long period of time.

The time of appearance and the amount of nonprecipitating antibody produced seemed to be dependent on the individual. For instance, B18, B20, and B23 gave their greatest response on day 171, while B21, B22, and B24 gave theirs on day 190. Rabbit B27 gave the most response on day 149, as did B28 which also showed a second peak on day 251. The magnitude of the response ranged from 2.13 for rabbit B24 to 4.05 for rabbit B28.

A second series of rabbits ("C" series) was subjected to the same

type of immunization and bleeding schedule in order to confirm the results given by the "B" rabbits. Rabbits C27, C28, C30, C36, and C38 were immunized and bled in the same manner as the "B" rabbits except for a shorter period of time and 2 extra bleedings.

The sera from the "C" series that were analyzed by the ABC and RID tests were collected on days 21, 42, 63, 84, 104, 119, 126, 132, and 146, with the exception of day 146 for rabbit C38. The results of these analyses are shown in Tables IV and V. As with the "B" rabbits, there were several "C" rabbits, C30, C36, and C38 that showed a concurrent increase and decrease in binding capacity and precipitating antibody concentration. However, rabbit C28 showed peak binding capacity at day 104 while the peak of precipitating antibody concentration was at day 42. Rabbit C27 seemed to show no real peaks of either binding capacity or precipitating antibody concentration.

The molar ratios, as described above, were calculated for the "C" rabbits also. Figures V and VI are graphs plotting these calculations. It can be seen that three of the rabbits, C27, C30, and C38, appeared to give a monophasic response. However, rabbits C28 and C36 gave a type of response that could be interpreted as diphasic, although not so definite as those exhibited by the three "B" rabbits.

Again, the time of appearance and amount of nonprecipitating antibody produced seemed to be a function of the individual rabbit. Rabbits C27, C28, and C36 showed a maximal molar ratio response on day 126, whereas C30 had its greatest response on day 146 and C38 showed the highest ratio on day 104. The magnitude of the greatest molar ratio for the "C" series of rabbits ranged from 2.56 of rabbit C38 to 4.35 of rabbit C28.

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TABLE IV. ABC (ug antigen bound/ml) and RID (ug precipitating antibody/ml) values for rabbits C27, C28, and C30, bled on days 21, 42, 63, 84, 104, 119, 126, 132, and 146.

Serum	ug Antigen Bound/ml	ug Precipitating Antibody/ml
C27-21	14	Undetectable
C27-42	81	144
C27-63	78	147
C27-84	119	136
C27-104	143	150
C27-119	137	136
C27-126	151	124
C27-132	121	114
C27-146	145	144
C28-21	64	180
C28-42	198	275
C28-63	185	227
C28-84	164	207
C28-104	376	214
C28-119	271	200
C28-126	323	166
C28-132	191	156
C28-146	238	160
C30-21	18	Undetectable
C30-42	162	290
C30-63	238	323
C30-84	350	330
C30-104	498	367
C30-119	475	340
C30-126	475	330
C30-132	356	250
C30-146	330	227

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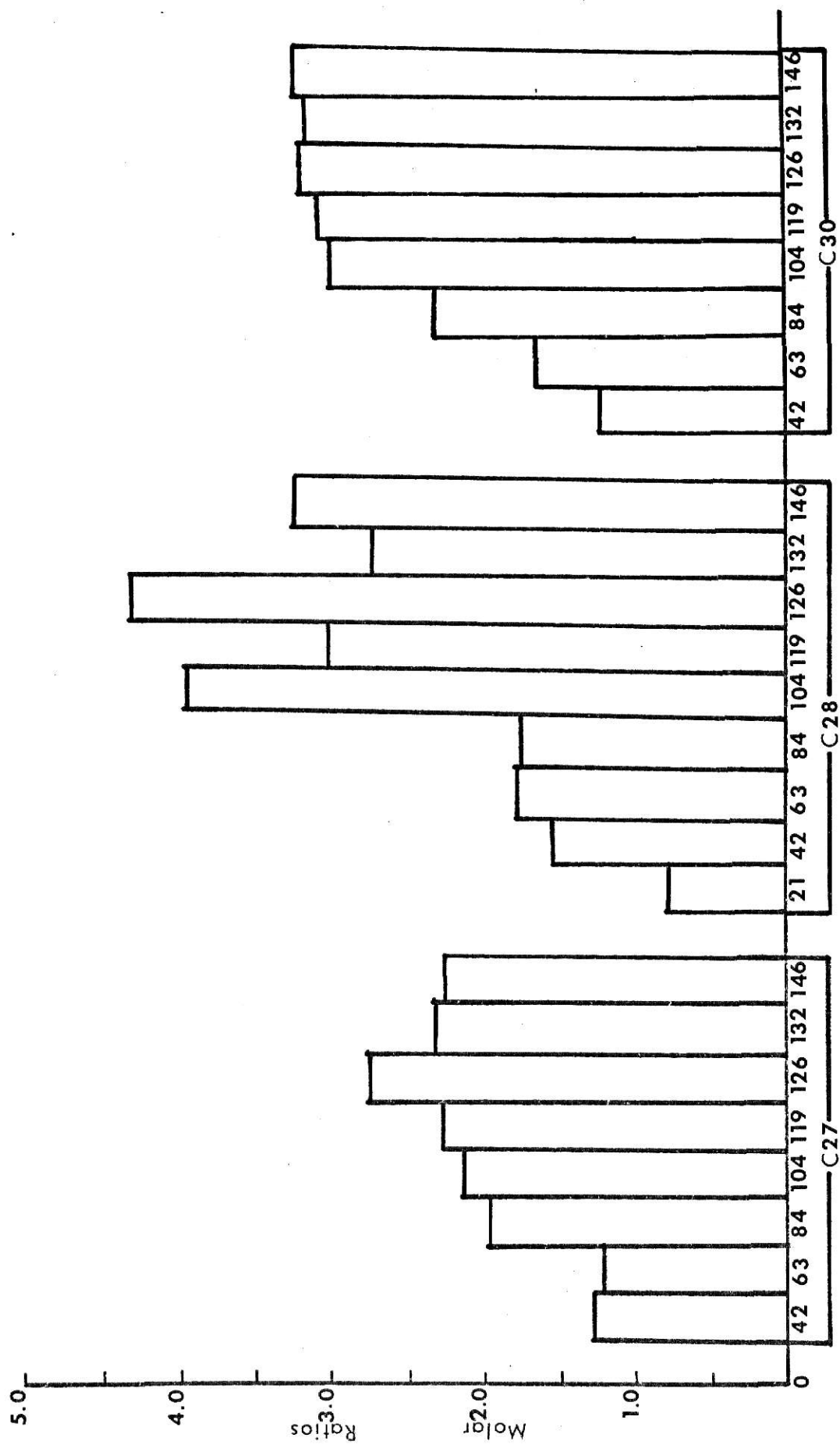
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TABLE V. ABC (ug antigen bound/ml) and RID (ug precipitating antibody per ml) values for rabbits C36 and C38, bled on days 21, 42, 63, 84, 104, 119, 126, 132, and 146.

Serum	ug Antigen Bound/ml	ug Precipitating Antibody/ml
C36-21	25	Undetectable
C36-42	99	178
C36-63	144	156
C36-84	158	227
C36-104	356	267
C36-119	271	223
C36-126	360	213
C36-132	221	166
C36-146	238	208
C38-21	15	Undetectable
C38-42	76	172
C38-63	264	320
C38-84	208	247
C38-104	200	176
C38-119	158	166
C38-126	142	132
C38-132	101	Undetectable

FIGURE V. Molar ratios of moles of antigen bound : moles of precipitating antibody per ml of serum for rabbits C27, C28, and C30, bled on days 42, 63, 84, 104, 119, 126, 132, and 146 (day 21 is included for C28).

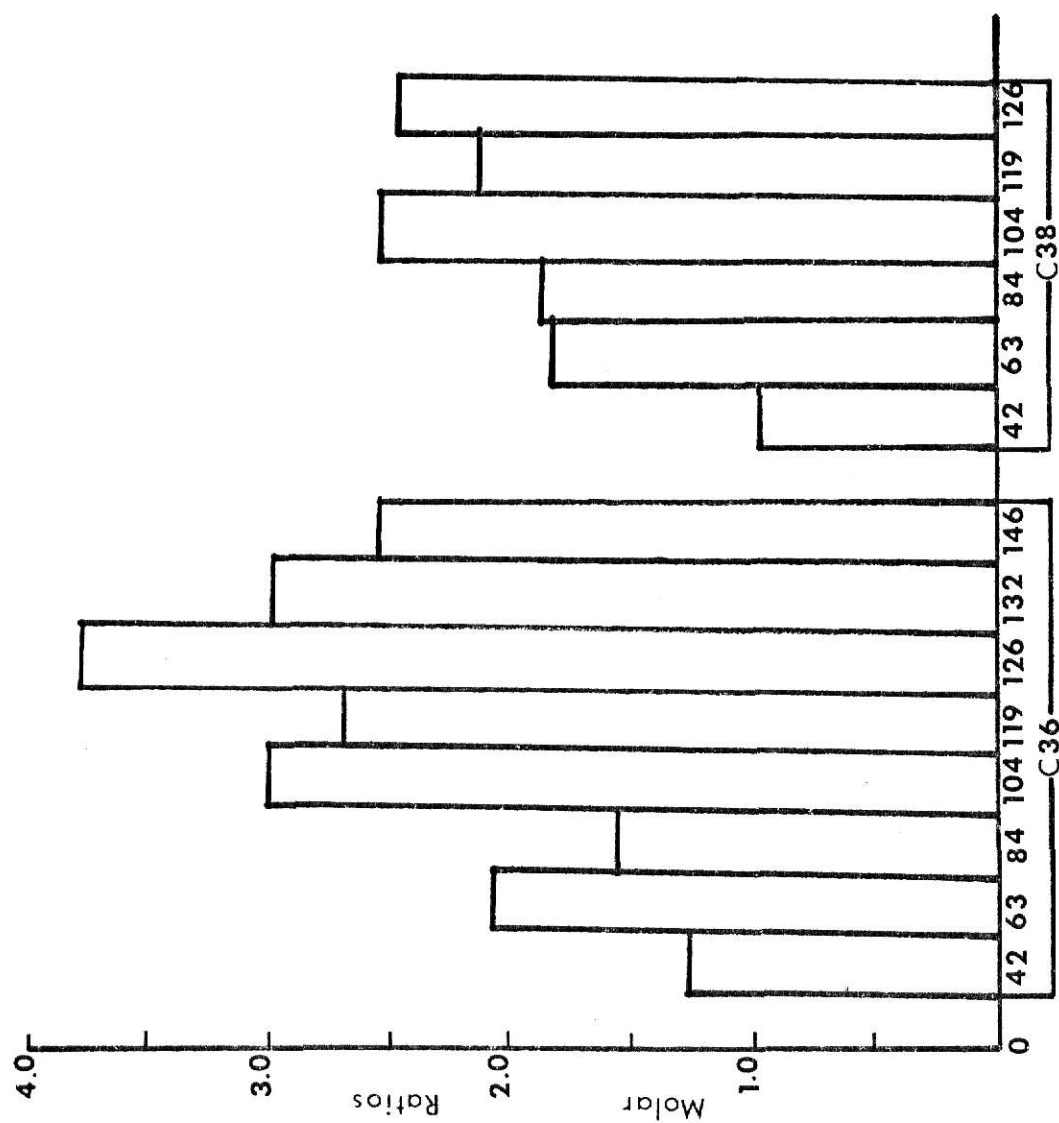
Graph of Molar Ratios of Antigen Bound: Precipitating Antibody per ml of Sera from Rabbits C27, C28, and C30



Rabbits and Days of Bleeding

FIGURE VI. Molar ratios of moles of antigen bound : moles of precipitating antibody per ml of serum for rabbits C36 and C38, bled on days 42, 63, 84, 104, 119, 126, 132, and 146 (except for days 132 and 146 for rabbit C38).

Graph of Molar Ratios of Antigen Bound: Precipitating Antibody per ml of Sera from Rabbits C36 and C38



Rabbits and Days of Bleeding

Both the "B" and "C" series of rabbits appeared to give the same types of results. Some of the rabbits exhibited a monophasic type of response, while others showed a diphasic one. In general the "C" rabbits gave a maximum molar ratio response before the "B" rabbits did. The reason for this result is not known, since both groups of rabbits were immunized and bled the same way. Both groups showed that the maximal molar ratio response was dependent on the individual rabbit and that the molar ratio changed throughout the course of immunization. The areas of high molar ratios (greater than 2.80:1.00) indicate that a significantly larger amount of antibody was being made that bound to antigen but would not effect precipitation.

The possibility for nonspecific protein-protein interaction to affect in vitro antibody-antigen interaction always exists when assaying whole serum samples. In order to study the binding and precipitating characteristics of the antibodies in the absence of nonimmunoglobulin serum proteins, the antibodies were purified using Sepharose immuno-adsorbent. These nonspecific interactions did not seem to affect the antigen-antibody interactions in the whole serum since after purification the binding values were not markedly increased or decreased (except for the decreased binding of the high molar ratio samples that was presumably caused by inactivation of the nonprecipitating fraction of antibodies) as shown by the molar ratios in Table VI.

Purification of specific antibodies in the sera was done as described previously in materials and method section. Propionic acid was used to elute the specific antibodies from the columns. The purified antibody preparations were then neutralized to pH 7.0 and dialyzed against BSB at 4°C overnight. The dialysis method just described was used for all

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TABLE VI. Molar ratios of whole sera and specifically purified antibodies.

Serum	ml Purified	Molar Ratio Before Purification	Molar Ratio After Purification
B21-212	35	2.84:1.00	-----*
B23-84	2	1.50:1.00	1.91:1.00
B23-171	35	3.39:1.00	2.80:1.00
B27-84	20	3.06:1.00	1.83:1.00
B27-171	35	2.89:1.00	2.65:1.00
B27-212	35	2.81:1.00	-----*
B27-42	21.5	1.09:1.00	1.44:1.00
Pool I	110	2.30:1.00	1.73:1.00
Pool II	110	2.90:1.00	2.09:1.00
Pool III	75	2.79:1.00	1.75:1.00
Pool IV	65	4.30:1.00	1.90:1.00
Pool V	120	1.79:1.00	1.28:1.00
Pool VI	120	3.31:1.00	1.75:1.00

Pool I = C28, days 42, 63, and 84.

Pool II = C28, days 104, 119, and 126

Pool III = C30, days 42, and 63

Pool IV = C30, days 126, 132, and 146

Pool V = C36, days 42, 63, and 84

Pool VI = C36, days 104, 119, 126, and 132

*Concentrations of precipitating antibody in samples B21-212 and B27-212 were too low to be detectable by the RID technique.

samples except for the pooled serum samples. The dialysis method after neutralization for the pooled samples was as follows: (1) dialysis against deionized water for about 24 hours; (2) dialysis against physiological saline for about 16 hours; (3) dialysis against BSB for about 8 hours; and (4) dialysis against 1 part borate : 4 parts physiological saline for about 16 hours. To check this method of dialysis as opposed to the original method, a simulation of the propionic acid effects on the molecules (unfolding of the antibody molecules) was done by dialyzing 4 ml of purified antibody preparation Pool V (pooled serum from C36, days 42, 63, and 84) and 4 ml of purified Pool VI (pooled serum from C36, days 104, 119, 126, and 132) against 1 M propionic acid overnight at 4°C. The two samples were then neutralized to pH 7.0 and dialyzed against BSB at 4°C for three days. ABC and RID tests were performed on the two samples. The data showing the ABC and RID values on the purified pools and how the recovery values were calculated are shown in Table VII. It can be seen that all or nearly all the precipitating antibody activity was recovered by the treatment described above. However, an appreciable amount of the binding activity appeared to be lost. This suggests that dialyzing against the BSB alone may not allow maximal correct folding of the antibody molecules, even when it is allowed to go for 3 days as opposed to the overnight method. However, this assumption excludes the possible deleterious effects which the propionic acid may have had on the antibodies.

The sera or pooled sera which were purified are listed in Table VI, along with the respective molar ratios before and after purification. These data indicate that especially those sera with a high molar ratio before purification had decreased ratios after purification. Calculations of the theoretical values for the binding and precipitating ability of the

TABLE VII. Data for purified Pools V (sera from rabbit C36, days 42, 63, and 84) and VI (sera from rabbit C36, days 104, 119, 126, and 132) that were subjected to propionic acid treatment, neutralized, and dialyzed according to the original procedure (against BSB at 4°C). This table also includes the recovery values for both the binding activity (ug Ag bound) and precipitating antibody concentrations (ug precipitating Ab).

Sample	ABC value per ug protein	RID value per ug protein	Protein conc.	Volume
Purified Pool V (before acid)	0.134 ug Ag bound	0.234 ug ppt Ab	385 ug/ml	4 ml
Purified Pool VI (before acid)	0.170 ug Ag bound	0.217 ug ppt Ab	396 ug/ml	4 ml
Purified Pool V (after acid)	0.104 ug Ag bound	0.245 ug ppt Ab	253 ug/ml	5.7 ml
Purified Pool VI (after acid)	0.138 ug Ag bound	0.254 ug ppt Ab	205 ug/ml	5.7 ml

The theoretical binding activity of Purified Pool V was 206 ug antigen (Ag) bound, contained in the 4 ml that were treated with acid, neutralized, and dialyzed. This was calculated by multiplying the ABC value given by the total ug protein in the 4 ml sample. The total precipitating antibody concentration of Pool V was calculated to be 360 ug precipitating antibody. The theoretical values of Pool VI were calculated to be 270 ug antigen bound and 344 ug precipitating antibody.

Values for actual recovery were calculated the same way, resulting in values of 156 ug antigen bound and 367 ug precipitating antibody for Pool V. The actual values for Pool VI were 180 ug antigen bound and 332 ug precipitating antibody.

Recovery percentages for both the binding and precipitating capacities were calculated by dividing the actual values by the theoretical values. These calculations show that 76% of the binding activity and 100% of the precipitating activity were recovered from Pool V. The recoveries for Pool VI were 67% for binding activity and 96% for precipitating antibody activity.

samples that were applied to the column and comparisons of these values to the actual values of the purified samples were done. These calculations took into consideration the volume applied to the column so that total binding and precipitating ability of the sample put onto the column could be correlated to the total binding and precipitating ability of the material eluted from the column. The binding and precipitating values for the purified samples were expressed per μg of protein and could be further converted into total binding and precipitating ability by knowing the total μg of protein present in the eluted sample. The actual values/theoretical values were expressed as recovery percentages.

The results of these calculations, shown in Table VIII, indicate that for all the purified sera or pooled sera with original molar ratios of greater than 1.50:1.00 there was a greater loss of binding activity than precipitating ability. There was always some loss of both activities through handling of samples and other manipulations but it was shown that those sera with original high molar ratios consistently lost more binding than precipitating activity. The amount of difference in loss of binding activity and loss of precipitating activity ranged from 4% (i.e., 4% more binding activity lost than precipitating activity, based on actual recovered activity/theoretical applied activity) to 43%, with an average of 22%.

Either the nonprecipitating portion of the specific antibody population was more tightly bound (i.e., had higher K_o values) and was not being eluted or the propionic acid elution was too rigorous for the nonprecipitating antibodies and was in some way denaturing them. Therefore, the following alternate method of elution was tried in order to recover functional nonprecipitating antibodies.

TABLE VIII. Recovery of antigen binding and precipitating activities following specific purification of antibody. Percentages are expressed as actual activity after purification divided by the theoretical activity applied to the column.

Serum	Molar Ratio Before	Percentage Binding Activity Recovered	Percentage Precipitating Activity Recovered
B23-84	1.50:1.00	43%	34%
B23-171	3.39:1.00	53%	64%
B27-42	1.09:1.00	44%	33%
B27-84	3.06:1.00	53%	90%
B27-171	2.89:1.00	35%	39%
Pool-I	2.30:1.00	45%	60%
Pool-II	2.90:1.00	47%	65%
Pool-III	2.79:1.00	43%	68%
Pool-IV	4.30:1.00	34%	76%
Pool-V	1.79:1.00	46%	64%
Pool-VI	3.31:1.00	30%	57%

Pool I = C28, days 42, 63, and 84.

Pool II = C28, days 104, 119, and 126.

Pool III = C30, days 42, and 63.

Pool IV = C30, days 126, 132, and 146.

Pool V = C36, days 42, 63, and 84.

Pool VI = C36, days 104, 119, 126, and 132.

The alternate elution method was done by using Tris-HCl buffer to maintain the column at a physiological pH and incorporating 8 M urea as a denaturing agent to remove the specific antibodies from the column, as described previously in materials and method section. The purified preparations were analyzed the next day by the ABC test as well as quantitated for protein content with the spectrophotometer. An appreciable amount (about 40%) of the "protein" dialyzed out. Some of the absorbance values for "protein", as measured before dialysis, may have included a tiny amount of the free hapten present in the solution which also absorbs greatly at 280 nm. The ABC test revealed very low binding activity per ug protein, although the ABC test on the 1:10 effluent material showed little binding (less than 6% of the iodinated antigen in the precipitate) except for one serum which had apparently overloaded the column. This indicated that much of the antibody had not been eluted from the columns.

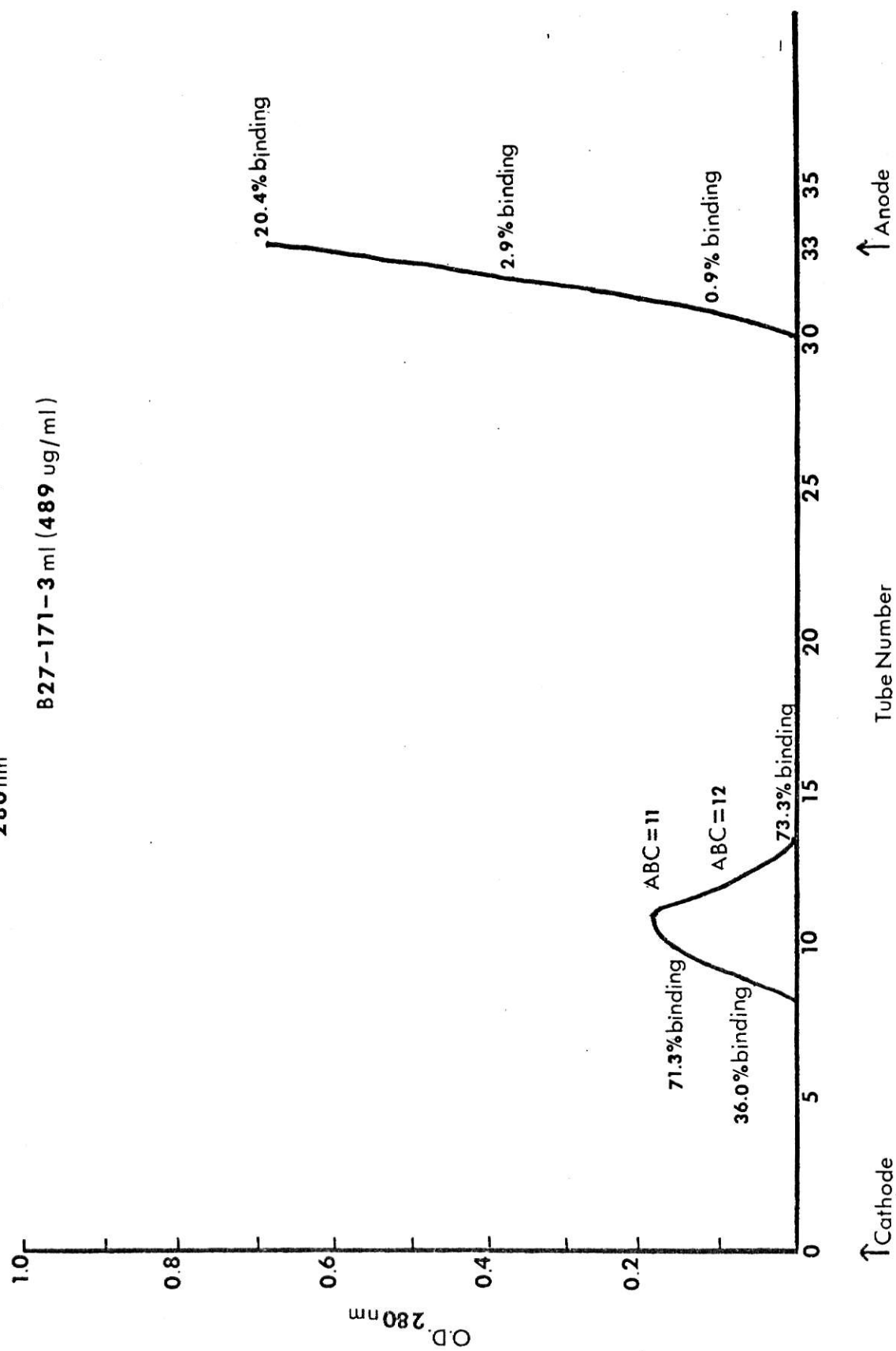
Specific antibody was eluted by using propionic acid on the same columns which had been left for 6 days at 4°C after having been washed with physiological saline to wash out the urea. This procedure eluted further protein from the column as monitored at 280 nm. Again some (ranging from about 30-60%) of the "protein" was lost after dialysis, but the binding ability per ug protein was much higher. For example, the ABC value observed was 0.167 ug antigen bound per ug protein, as compared to an ABC value of 0.015 per ug protein for the antibody eluted with the Tris-HCl buffer containing 8 M urea.

However, serum from rabbit B27 day 171 had a high molar ratio that was not so adversely affected by the purification procedure. This sample was used in the preparative electrophoresis since its ratio changed only from 2.89:1.00 to 2.65:1.00. Figure VII shows that two peaks resulted in

FIGURE VII. Preparative electrophoresis results after elution of protein with lactate barbital buffer. The purified antibody preparation used was B27-171. The amount electrophoresed was 3 ml with a protein concentration of 489 ug/ml. The binding percentages or ABC values indicated are the results obtained for each of the undiluted samples.

Graph of O.D. $_{280\text{nm}}$ VS Tube Number for Preparative Electrophoresis

B27-171-3 ml (489 ug/ml)



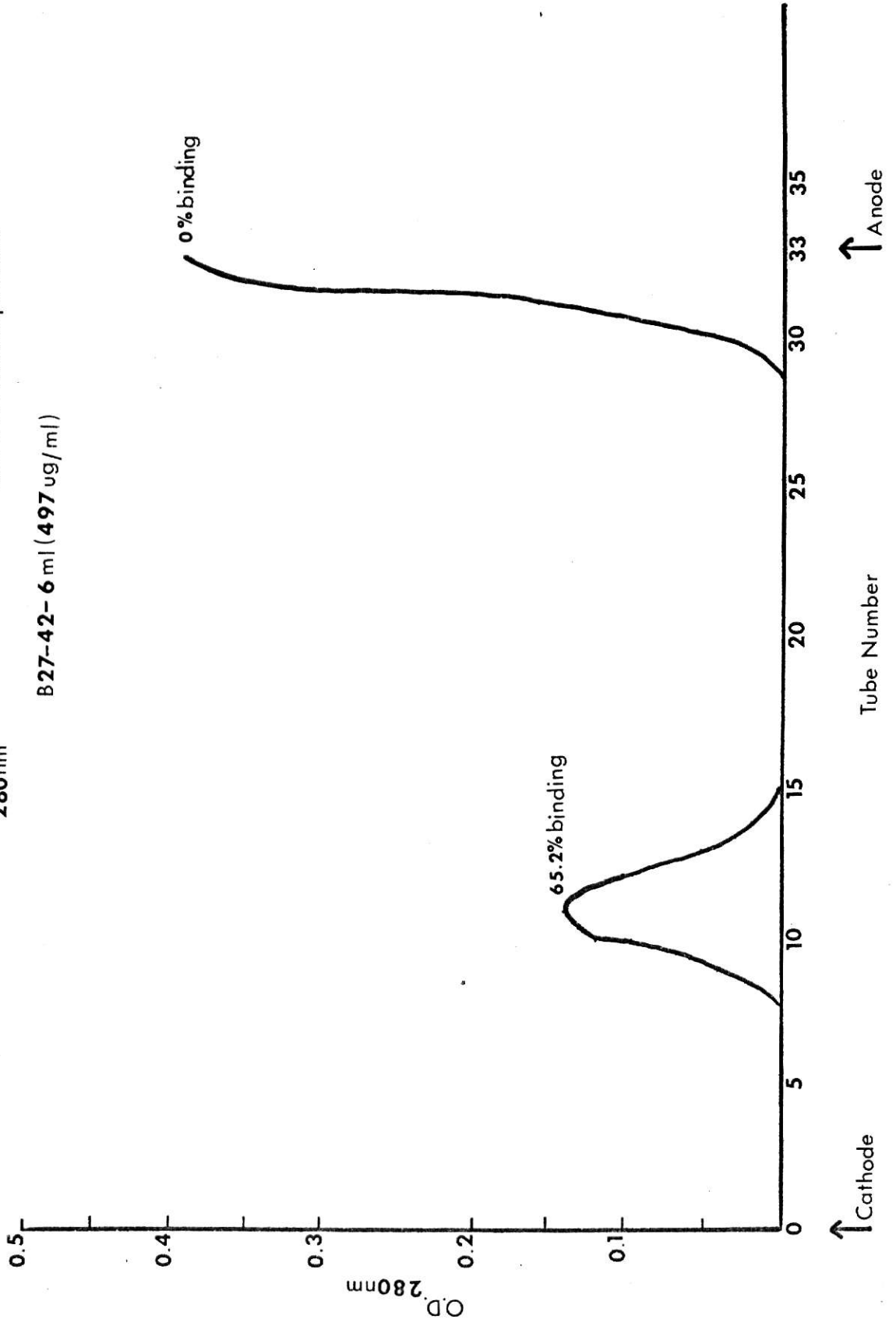
the electrophoresis run, one in the region expected for gamma globulin migration (tubes 9-13) and one at the anode end (tubes 31-33). Both of these peaks were analyzed by the ABC test; the first peak contained most of the binding activity, as expected, but the second peak showed 20% binding of the iodinated antigen (corrected for NSB) in the undiluted form. This suggested that some type of antibody may be present at the anodal end. A repetition of these results using more highly concentrated purified antibody was desired so that an ABC and RID value could be obtained. But, as explained previously, this was not possible because a better technique had not been developed to keep the nonprecipitating antibodies functional during purification.

A purified antibody sample having a low molar ratio (B27-42) was electrophoresed as a comparison with the above results. The objective was to determine if the unusually fast migrating antibody was detectable in a sample with a low molar ratio. Six (6) ml of this preparation was applied to the block and electrophoresed. Figure VIII shows that this sample had the expected peak at tubes 9-13 and also a smaller peak at the anodal tubes 31-33. As assayed by the ABC test, there was binding activity in the first peak as expected, but no activity was present in the second peak. It is noteworthy that some of the O.D._{280 nm} absorbing material present in the second peak was observed to dialyze out during the dialysis against BSB in several previous electrophoretic runs. This loss of absorption was assumed to be some type of electrophoresis product which absorbed at 280 nm but which was not protein, since it passed the dialysis bag. Therefore both dialyzed and undialyzed samples were used for this series of ABC tests on the anodal peak, as well as for the following blank run. Either dialyzed or undialyzed samples used for the

FIGURE VIII. Preparative electrophoresis results for purified antibody preparation B27-42. Six (6) ml with a protein concentration of 497 ug/ml was used. The graph shows the amount of protein eluted with lactate barbital buffer. The binding percentages are those obtained from ABC tests done on undiluted samples contained in tubes 11 and 33.

Graph of O.D._{280nm} VS Tube Number for Preparative Electrophoresis

B27-42-6 ml (497 ug/ml)



ABC tests gave the same results. Also tubes 31-33 of this sample were concentrated by pervaporation to check whether low protein concentration was the reason for the absence of binding activity.

As a check on the rapidly migrating antibody shown in the high molar ratio sample, a blank electrophoresis run was made using just the lactate barbital buffer as the sample to see if there was some type of dialyzable electrophoresis by-product being formed which absorbed at 280 nm. A 5 ml sample was electrophoresed and, as shown in Figure IX, only one peak, at the anode end, was observed. Some of the material comprising the peak was dialyzed and it subsequently lost its absorbance at 280 nm. Neither the dialyzed nor the undialyzed samples showed binding activity in the undiluted state.

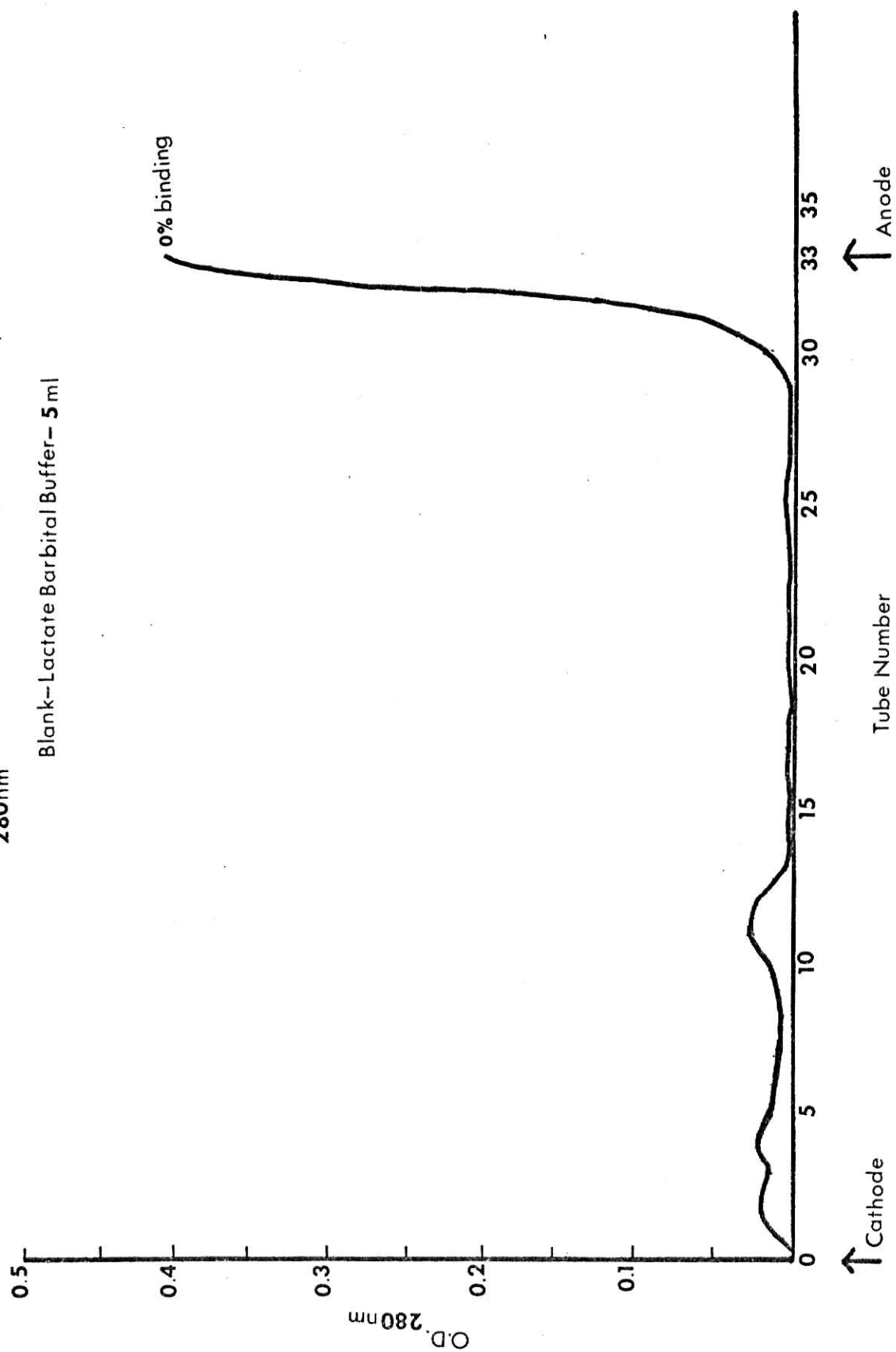
These preliminary data indicate that there was a unique type of antibody population present in the purified antibody preparation with a high molar ratio which migrated relatively fast. This fast component did not appear to be in the purified sample that had a low molar ratio. There also seemed to be some type of electrophoresis by-product formed in this system which absorbed at 280 nm but which was dialyzable and did not have the ability to bind antigen.

FIGURE IX. Preparative electrophoresis results for 5 ml of
lactate barbital buffer.

The binding percentage given is that obtained
from an ABC test done on the undiluted sample.

Graph of O.D. $_{280\text{nm}}$ VS Tube Number for Preparative Electrophoresis

Blank-Lactate Barbitol Buffer- 5 ml



DISCUSSION

The purposes of this study were to evaluate the nonprecipitating anti-hapten antibody response elicited during prolonged immunization of rabbits, to purify the specific anti-hapten antibodies, and to do some preliminary characterization of these nonprecipitating antibodies.

The antigen system used in this study was carefully selected to insure that only anti-hapten antibodies were being analyzed. The immunizing antigen, KLH-R⁴N, probably caused the formation of antibodies with three specificities: (1) anti-KLH; (2) anti-KLH-R⁴N; and (3) anti-R⁴N. Since the test antigen used was BSA-R⁴N, either antibodies with specificities directed against the hapten, R⁴N, or those against KLH-R⁴N with high K_o values were assayed. This approach of studying anti-hapten nonprecipitating antibodies was similar to that of Klinman *et al.* (23) in their equine anti-Lac studies. They used KLH-Lac as an immunizing antigen and HSA-Lac as the test antigen. The idea of using anti-hapten antibodies to study nonprecipitating antibodies is advantageous because the antigenic determinants against which they are made are chemically defined. This is in contrast to antibodies made in response to heterogeneous antigens, such as BSA. The antigenic determinants responsible for the formation of anti-BSA antibodies are heterogeneous, in contrast to the homogeneous determinants of haptens. This is because BSA is a long polypeptide with many different amino acids and resultant peptides that can act as antigenic determinants.

The reason for choosing R⁴N as the hapten in this study was that it is a hapten possessing a net positive charge. Haptens with either a net negative charge or a net charge of zero will, when coupled to BSA, be

precipitated by 50% saturated ammonium sulfate used in the ABC test. It was desirable to use a hapten which, when coupled to BSA, would remain in the supernatant if it were not bound to specific antibodies because the ABC test was an integral part of this whole investigation.

There are two possible antibody responses that may result after stimulation of an animal with a foreign antigen. One of these is the production of a homogeneous antibody population (34), probably due to the activation of a single clone of antibody-producing cells. The other possibility is the production of a heterogeneous population of specific antibodies (15). The explanation for the heterogeneous antibody response may be that two clones of antibody-producing cells are activated and subsequently produce antibodies with the same specificities but with different precipitating capabilities.

As can be seen from Figures II-VI, the antibody response observed in this study appears to result in the production of a heterogeneous population of specific anti-hapten antibodies. This is evidenced by the change in molar ratios throughout the course of immunization shown by these data. All of the rabbits tested exhibited this change in molar ratios. If the response had resulted in the production of a homogeneous population of antibody, the molar ratios would not have changed because any increase in binding ability would have been accompanied by a concomitant increase in precipitating ability. In this study, however, there were instances of increases in binding ability which were not accompanied by increases in precipitating ability, as shown in Figures II-VI by the increased molar ratios and in Tables I-V by the increased amounts of antigen bound per ml of serum. These increases in binding ability indicate the presence of nonprecipitating antibody.

The immunizing and testing antigens were made by coupling many identical determinants to each protein molecule to be certain that the nonprecipitability that was observed was due to a function of the antibody and not of the antigen. This procedure effectively made each molecule multivalent with respect to a single determinant site. The use of this type of antigen system circumvented the criticism that the observed nonprecipitability may be due to the structure of the antigen and not due to a unique characteristic of the antibody involved. The fallacy of using heterologous antigens in studying nonprecipitating antibodies can be illustrated with BSA as an example. Each BSA molecule has many different antigenic determinant sites. Any one, several, or all of these determinant sites may elicit the antibody response in the animal. Nonprecipitability of the antigen would be observed if just one of the sites were responsible for causing the antibody formation against the BSA molecule and if that one determinant site were present only once on the BSA molecule. The reason for the nonprecipitability is that the lattice structure necessary for precipitation could not occur. Only one antibody molecule could bind with each antigenic site although two antigen molecules could be bound per antibody molecule. In contrast, one antibody molecule must be bound to two antigen molecules, which in turn must be bound by at least two antibody molecules, in order to form the lattice framework of precipitation. The nonprecipitability observed in this study must have been due to the structure of the antibody because the antigens used had many of the same determinant sites (R4N) on their surfaces and could have formed the lattice needed for precipitation. Poor choice of antigen is a criticism that can be leveled against studies of nonprecipitating antibodies done by others who used heterologous

antigens. Some of the heterologous antigens that have been erroneously used to study nonprecipitating antibodies include egg albumin (12, 15, 18, 27, and 29), bovine serum albumin (5, 10, 30), bovine plasma albumin (12), and human serum albumin (5, 10, and 13).

In this study, the time of appearance of an appreciable amount of nonprecipitating antibodies was found to be variable as can be seen by the increases in the molar ratios shown in Figures II-VI. The sera of three of the "B" series rabbits, for example, contained nonprecipitating antibodies, as indicated by a relatively high molar ratio (greater than 2.50) at day 171. This same phenomenon was shown by 2 "B" series rabbits at day 190, 1 rabbit at day 212, 1 rabbit at day 84 (also showed a second peak at day 212), and 1 rabbit at day 149. In the "C" series of rabbits, four out of five animals first had a relatively high molar ratio on day 104. The other rabbit responded on day 126. It is somewhat difficult to make comparisons between the time of appearance of nonprecipitating antibodies in this study and the time interval seen in other studies since it is not clear from much of the literature in this area as to just what the time interval was between the beginning of injecting the animals and the appearance of nonprecipitating antibodies. For example, Heidelberger et al. (15), Gitlin et al. (13), and Pappenheimer (29) all reported that the nonprecipitating antibodies appeared early in the course of immunization, but they did not report the exact time of appearance. Heidelberger (15) further reported that these nonprecipitating antibodies remained in the serum even after prolonged immunization. Unfortunately, he did not quantitate the amount of nonprecipitating antibodies or indicate when they were observed so that a comparison could be made of when the maximum amount of these antibodies was reached. There

were a few researchers that showed when the nonprecipitating antibodies appeared. Feinberg (10) found nonprecipitating antibodies in the serum of rabbits which had been immunized with either BSA or HSA emulsified in complete Freund's adjuvant between 8 and 16 weeks, with a maximum at 12 weeks (84 days). Klinman et al. (23) reported the presence of nonprecipitating antibodies in the serum of a horse immunized with KLH-Lac, emulsified in incomplete Freund's adjuvant, after 90 days. Margni (27) used a serum sample collected after 70 days of immunizing a rabbit with antigen emulsified in complete Freund's adjuvant, and found nonprecipitating antibodies. A possible explanation for the results of these early studies, which indicate the presence of nonprecipitating antibodies in the serum at the early stages of immunization, may be the lack of adjuvant or the use of a less effective adjuvant during the injection of the antigen. Testing this hypothesis might be done by injecting antigen both with and without adjuvant although this was not done in the present study.

The method of detecting nonprecipitating antibody was to use the ABC test to detect the binding ability of a sample and to use a modification of the radial immunodiffusion to detect precipitating antibody. The ABC test worked quite well without much altering of the classical technique, but the RID procedure took more trial and error experimentation until the appropriate concentrations of antigen and amounts of sample to be added to the agar were found. The RID method then became an invaluable tool for this study. One main advantage of this method of detecting precipitating antibody is that it used much less sample to get the results than the quantitative precipitin test. This is a very important consideration, especially for limited volumes of serum. These two test and calculations of the molar ratios were done to show that this combination could be used

to detect nonprecipitating antibodies. A combination of both of these sensitive techniques was advantageous because it required the use of small sample volumes, in the range of 2-3 ml. A test capable of detecting primary antigen-antibody interactions (ABC test) that was not dependent on a previous test to detect secondary interactions (precipitin test) was used in this study. This approach was in contrast to those used in other studies. In many previous studies nonprecipitating antibodies were detected in the supernatants resulting after either 1) serially absorbing the precipitating antibody with small amounts of antigen (3, 12, 18, and 27), or 2) after quantitative precipitin test (15), or 3) after the quantitative isotope precipitin technique (10 and 30). The methods previously used to detect the nonprecipitating antibodies in the above supernatants include the anti-globulin test (10 and 38), precipitation of the bound radiolabelled antigen with 50% saturated ammonium sulfate (30), coprecipitation (18), specific removal from the supernatant using columns made with polymerized antigen (27), or testing for passive hemagglutination activity (12). In one case, in which precipitating antibodies were not demonstrable (5), the nonprecipitating antibodies were detected by using the ABC test. Comparison of values obtained by using the method employed in this study to the values obtained by using the previous methods cannot be made because these sera were not assayed by those other methods. All that is known from this study is that this method worked as shown by the changes in molar ratios shown in Figures II-VI and that this approach has several theoretical and practical advantages.

The purification procedure proved to be the real difficulty for this study. During one of the few initial purifications, an antibody (thusly

called due to the 20% antigen binding ability observed using the ABC test) was detected that exhibited a very rapid migration in the preparative electrophoresis, as shown in Figure VII. To purify the pooled sera samples, get sufficient protein concentration for preparative electrophoresis, and attempt to characterize this rapidly migrating antibody (assuming it would show up in those purified pooled sera which demonstrated high molar ratios as it had before) were the next steps in the study. However, after the purification of these pooled sera, those sera possessing high molar ratios prior to purification had quite decreased ratios after purification (see Table VI). During the initial purifications of the unpooled serum samples (Table VI), sera with the originally high molar ratios lost these high ratios or had them somewhat decreased. However, the initial loss was not so great as that incurred during the purification of the pooled serum samples. It appeared that there was some part of the purification procedure that seemed to consistently inactivate the nonprecipitating fraction, as illustrated by the recovery percentages calculated for both the binding and precipitating activity in Table VIII. The nonprecipitating fraction was either inactivated and rendered nonfunctional or it was not being eluted from the column. An alternate method of elution was done using Tris-HCl buffer to maintain the column at a physiological pH and incorporating 8 M urea as a denaturing agent. However, as already mentioned in the results section, less than 6% of the iodinated antigen appeared in the precipitate in the ABC test, indicating that this method of elution was not satisfactory. It is very clear that some further experimentation is necessary in this area of antibody purification and in the study of nonprecipitating antibodies.

A rapidly migrating protein was isolated from a purified sample with

a high molar ratio. This protein had specific binding activity for the antigen. It appeared to be an unusual and previously unrecognized antibody with unique physicochemical properties because it migrated faster than any antibody previously reported. This type of antibody appearing in sera with an appreciable amount of nonprecipitating antibodies is in contrast to the previous reports on the migrating behavior of nonprecipitating antibodies. Studying rabbit nonprecipitating antibodies, Feinberg (10) found evidence that suggested that the nonprecipitating antibody migrated as a gamma globulin. Patterson (31) found that the canine nonprecipitating antibody he was investigating migrated as a beta-2 globulin. Perhaps the fraction found in the present study had been missed prior to this time since it would migrate faster than albumin during the electrophoresis of whole serum and usually no more protein is anticipated to migrate faster to the anode than albumin. If this rapidly migrating protein were present in sufficient concentration in the whole serum, it might be detectable using cellulose acetate electrophoresis. The only drawback with that type of electrophoresis is that the unique peak, if present, could not be used for further characterization. However, this method, since it uses very little serum, would be a good screening procedure if it were sensitive enough to detect that peculiar fast migrating fraction.

There may be some important medical aspects of nonprecipitating antibodies. Patterson et al. (31) have found that nonprecipitating antiserum caused the antigen to be initially retained in the circulation of a passively sensitized animal (in this case guinea pigs initially injected with iodinated BSA, followed by the antiserum). In a six day elimination experiment, those guinea pigs which received the nonprecipitating antiserum showed retention of the soluble complexes during the first

24 hours, followed by an elimination rate equal to that of those animals receiving precipitating antiserum and faster than for the elimination of the iodinated antigen itself. It was thought that circulation of soluble complexes may be important in vascular injury (16). This may be a reasonable proposal since Pincus et al. (32) showed that rabbits with a nonprecipitating antibody response were those that subsequently developed chronic glomerulitis.

It is possible that the type of immunization schedule employed in this study (prolonged stimulation) may simulate conditions of chronic disease states. It might be of value to look for nonprecipitating antibodies in animals with those types of conditions.

In summary, this study evaluated the production of nonprecipitating antibodies against the hapten R₄N in rabbits that received prolonged immunizations. The nonprecipitating antibodies were detected by using a combination of the Farr technique and radial immunodiffusion, followed by the calculation of the molar ratios of moles of antigen bound : moles of precipitating antibody. Those samples that had molar ratios of greater than 2.80 were considered to contain appreciable amounts of nonprecipitating antibodies. Sera were purified using solid immunoabsorbent columns and the sera were assayed both before and after purification by the Farr method and radial immunodiffusion. A purified sample with a high molar ratio (greater than 2.60) was subjected to preparative electrophoresis and was found to contain a fast migrating protein which exhibited binding ability. Neither a purified sample with a low molar ratio (less than 1.50) nor buffer alone showed binding at the same position at which binding was detected in the sample with a high molar ratio. This fast migrating protein, found in the sample with an appreciable amount of nonprecipitating

antibodies but not in the low molar ratio sample or the control electrophoresis run, suggests that this previously unrecognized protein may be associated with nonprecipitating antibodies. The protein showed specific binding activity which suggests that it is an antibody. However, more characterization study must be done on this protein to elucidate its role in the phenomenon of nonprecipitability of antigen.

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A STUDY OF
NONPRECIPITATING ANTIBODIES

by

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This study was made to evaluate the production of nonprecipitating anti-hapten antibodies in two groups of rabbits that received a prolonged series of immunizations.

Sera containing antibodies made against the hapten p-aminophenyl-N-trimethyl ammonium chloride (R^4N) were assayed for antigen binding capacity (ug antigen bound per ml serum) by a primary binding test and for concentration of precipitating antibodies by a precipitation test done in agar. Molar ratios of moles of antigen bound per ml serum : moles of precipitating antibody per ml serum were then calculated for each serum. Sera showing a molar ratio of greater than 2.80 were considered to contain nonprecipitating antibodies.

Purification of serum samples was accomplished by using a solid immunoabsorbent. The specifically purified antibodies were then eluted with propionic acid, neutralized, dialyzed, and quantitated for their binding and precipitating ability.

A purified sample with a high molar ratio was used in preparative electrophoresis. This sample contained a fast migrating protein that exhibited binding ability. Another purified sample with a low molar ratio was also electrophoresed but showed no binding in the position in which binding had been detected in the sample with the high molar ratio. A control electrophoresis run, using only the buffer used in the system, showed no binding in the same position. This unusually fast migrating antibody was thought to be relevant to this study because this protein was found in the serum which contained an appreciable amount of nonprecipitating antibodies as shown by the high molar ratio.

Another finding of this study was that the molar ratios of those sera

with high molar ratios before purification were dramatically decreased after elution of the specific antibodies with propionic acid in all cases except two. This decrease in molar ratios resulting from the inability to recover the nonprecipitating antibody activity suggested that either the nonprecipitating antibodies were not being eluted or that the propionic acid was somehow altering their ability to react with the antigen. An alternate method of elution at physiological pH using 8 M urea as a denaturing agent was tried but this was not effective in eluting the specific antibodies.

In summary, the rabbits gave a heterogeneous anti-hapten antibody response after prolonged immunization. This type of response was manifested by the presence of both precipitating and nonprecipitating antibodies in the serum. An unusually fast migrating, not previously described, type of antibody was present in a specifically purified sample that had an appreciable amount of nonprecipitating antibodies, as shown by a high molar ratio.