FACTORS AFFECTING 7S AND 17S ANTIBODY CONCENTRATIONS AND AFFINITIES IN CHICKENS

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By

Karen Yamaga

Dissertation Committee:

Albert A. Benedict, Chairman
John B. Hall
Mendel Herzberg
Douglas C. Vann
Kerry T. Yasunobu

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ABSTRACT

The formation of different immunoglobulin classes and binding constants were determined during the immune response in chickens injected with highly substituted dinitrophenyl-bovine gamma globulin (DNP-BGG). A comparison between intravenous immunization and intramuscular injections with adjuvants was made. Antisera were assessed for hemagglutination and precipitating anti-DNP antibody, and the presence of 7S and 17S antibodies was detected by radioimmunoelectrophoresis (RIE). The binding constants were measured from 7S and 17S globulin preparations using extensive equilibrium dialysis determinations.

Following an intravenous injection of DNP-BGG, the primary response was characterized by a transient synthesis of low amounts of 7S and 17S anti-DNP antibodies. A single intravenous injection did not induce sufficient amounts of antibody to perform equilibrium dialysis studies. However, a second intravenous injection resulted in a more vigorous production of both classes of antibodies. Hemagglutinating titers were high and precipitins were present within 4 days after the second injection. Furthermore, the 17S antibody was detected for at least 4 months by RIE.

In contrast to the response to intravenous injections, the response of chickens given a single intramuscular injection of antigen in either Freund's complete (FCA) or incomplete (FIA) adjuvants was characterized by an initial synthesis of 7S and 17S antibodies followed by the exclusive and persistent production of 7S antibodies. Hemagglutinating titers were low even after 2 intramuscular injections of antigen in

adjuvant. After a single injection of antigen in FCA with doses ranging from 0.02 to 20 mg, binding of the 7S anti-DNP antibody was detected by RIE for at least 490 days.

As a result of either intravenous or intramuscular injections of DNP-BGG, the anti-BGG responses were more transient in nature than the anti-hapten responses. After two intravenous injections little or no anti-BGG antibodies were detected by RIE one month later. Persistent synthesis of 7S anti-BGG antibodies generally required 2 intramuscular injections of DNP-BGG in FCA.

Injections of low doses of antigen in either FCA or FIA in chickens induced 7S antibodies of high affinity ($-\Delta F^\circ$ > 10 kcal/mole) by 3 months. The non-linearity of the Sips plots generated from the binding data indicated that a shift in the distribution of antibody affinities occurred due to the production of high affinity antibodies. In birds immunized with FCA the high affinity antibodies had restricted heterogeneity as indicated by heterogeneity indices of 1. Chickens given multiple intravenous injections, however, failed to produce high affinity antibodies. Despite altering the priming dose, the interval between injections and the number of injections, chickens injected intravenously produced 7S antibodies with $-\Delta F^\circ$ values that were consistently less than 10 kcal/mole. Sips plots of the binding data indicated that the antibody affinities were distributed in a normal array. It appears that at least two important conditions are required for eliciting high affinity antibody, namely, limited concentrations of antigen and the adjuvant effects.

Nearly identical affinities and heterogeneity indices were obtained for both the 7S and 17S antibodies isolated from 12 individual chickens given 2 intravenous injections of antigen. Thus, intravenous injections of antigen failed to elicit high affinity 7S and 17S antibodies. The conditions required to generate circulating 17S antibody of high affinity are not known.

In chickens given 2 intravenous injections of DNP-BGG, more 7S antibody was produced when the interval between intravenous injections was short than when the interval was longer. Intravenous injections apparently failed to induce large numbers of long-lived memory cells. Less antibody was synthesized in birds given either low (0.02 mg) or high (20 mg) doses of priming antigen than in birds injected with intermediate doses.

Specifically purified low affinity 7S antibodies had valences of less than 2, whereas a valence close to 2 was found for antibodies of higher affinities. Similarly 17S antibody of low affinity gave valences less than 10. Obtaining valences less than the expected value is best explained on the basis of contamination of the preparation with non-binding proteins or the presence of antibody with very low affinity.

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Commonly used abbreviations in this study:

"a": Sips heterogeneity index

B-cell: bone-marrow or bursa-derived lymphocyte

BGG: bovine gamma globulin

DNP: dinitrophenyl

DNP-OH: dinitrophenol

$-\Delta F^{\circ}$: negative standard free energy change expressed in kcal/mole

FCA: Freund's complete adjuvant

FIA: Freund's incomplete adjuvant

HSA: human serum albumin

Ig: immunoglobulin

K_o: intrinsic association constant expressed in M^{-1}

KLH: keyhole limpet hemocyanin

PFC: plaque forming cell

PHA: passive hemagglutination

RIE: radioimmunoelectrophoresis

7S Ig Fc: crystallizable fragment produced by papain digestion of 7S Ig consisting of heavy chains with class-specific determinants

T-cell: thymus-derived lymphocyte

I. INTRODUCTION

The distinguishing characteristic of immunoglobulins as a unique class of proteins is their heterogeneity. Based on antigenic markers, immunoglobulins form classes and subclasses (isotypes), differ between individuals of the same species (allotypes) and are unique for each clone of antibody-producing cells (idiotypes). Based on functional characteristics, antibodies bind specifically to an antigenic determinant with a given affinity; and exhibit effector reactions, such as the ability to attach onto membrane receptors and to fix complement. Some of these properties have been recognized in chicken immunoglobulins. There are 3 classes of chicken immunoglobulins: the low molecular weight and high molecular weight immunoglobulins, referred to as "7S" and "17S Ig," respectively (reviewed by Benedict & Yamaga 1974), and a recently identified IgA-like immunoglobulin (Lebacq-Verheyden et al 1972; Orlans & Rose 1972; Bienenstock et al 1972). A summary of the structural and biological properties of the 7S and 17S Ig is presented in Table I. Only a few characteristics of IgA-like Ig are known. Intestinal IgA-like Ig sediments at 11.9S to 16.2S (Bienenstock et al 1973). Small amounts are present in normal chicken serum (Leslie & Martin 1973) predominantly in polymeric form although monomeric IgA-like Ig also is detected. Chicken allotypic markers have been identified (Skalba 1964, 1966; Schierman & McBride 1968; David et al 1969) but the precise location of these determinants on the immunoglobulin molecule is not known. Idiotypic markers have not been described. The functional characteristics of chicken immunoglobulins have received considerable attention especially regarding the high-salt dependent precipitin

TABLE I

SOME PROPERTIES OF CHICKEN 7S AND 17S IMMUNOGLOBULINS[a]

Property	7S Ig	17S Ig	Reference
Molecular weight (x 10^{-3})			
Ig	170	890	Hersh et al (1969);
H chain	60.5	62.6	Leslie & Clem (1969b);
L chain	23.7	23.9	Kubo (1970)
Fab	55.7		
Fc	56.9		
Concentration in "normal" serum (mg/ml)	2.7 (young) 5.3 5.9 7.5 (> 1 year old)	0.7	Cooper et al (1969); Leslie & Clem (1970); Van Meter et al (1969); Tan (1974)
Reduction at neutral pH in the absence of a dissociating agent	Release of free L chains	Release of free L chains	Dreesman & Benedict (1965a); Benedict (1967b)
Lability of disulfide bonds to mild reduction	H-L > H-H	probably H-L > H-H	Kubo (1970)
Enzymatic hydrolysis			
Papain	Fab; Fc		Dreesman & Benedict (1965b);
Pepsin	Fab' (major); some $F(ab')_2$; and pFc		Kubo (1970)
Trypsin	tFab; tFc; $tF(ab')_2$		
Aggregation in 1.5 M NaCl (high salt)	Yes, ($S_{20,w}$=14S)		Hersh & Benedict (1965)
Immune precipitation in high salt	Enhanced	Decreased	Wolfe (1942); Kubo (1967)
L chain N-terminal amino acid sequence	Mostly unblocked λ^b (anti-DNP)		Kubo et al (1971); Hood et al (1970)
H chain N-terminal amino acid sequence (anti-DNP)	Resembles $V_H III^c$		Kubo et al (1971)
Total carbohydrate (%)	5.5	6.6	Acton et al (1972)
Metabolic half-life (days)	3.0 (neonatal) 1.5 (adults) 4.3 (adults) 4.1 (adults)	1.7 (adults)	Patterson et al (1962); Westman & Olson (1964); Leslie & Clem (1970)
Complement fixation	Guinea pig C (-) Chicken C (+)		Bushnell & Hudson (1927); Rice (1947)
Antibody in passive cutaneous anaphylaxis	Homocytotropic; sensitizes young birds optimally		Celada & Ramos (1961); Kubo & Benedict (1968); Conway et al (1968)
Antibody in reverse passive Arthus reaction	Sensitizes young birds optimally. Guinea pig poorly sensitized.		Luoma & Benedict (1974)

[a] Table modified from Benedict and Yamaga (1974).

Amino acid position:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
[b]	[ ]	-	[ ]	-Ala	-Leu	-Thr	-Gln	-[ ]	-Pro	-Ala	-Ser	-Val	-Ser	-Ala	-Gln	-Leu	-Gly	-Glu	-Thr	-Val	-Ser	-Leu	-Thr	-Cys-
[c]	Ala	-Val	-Thr	-Leu	-Asp	-Glu	-Ser	-Gly	-Gly	-Gly	-Leu	-Gln	-Thr	-Pro	-Gly	-Gly	-	-	-Leu	-	-	-Leu	-Val	-Cys-

reaction, complement fixation and immediate-type hypersensitivity (see Benedict & Yamaga 1974), but relatively little information is available on the binding properties of avian antibodies. It is known that chicken 7S antibody against the dinitrophenyl (DNP) groups has 2 binding sites (Gallagher & Voss 1969; Voss & Eisen 1972) and that the specificity of the anti-DNP sites, as determined by the relative binding of various nitrobenzyl compounds, is about the same as that found in mammalian anti-DNP antibody (Gallagher & Voss 1969).

During the immune response different populations of immunoglobulins identified by antigenic and functional properties may appear. Many factors determine the development of these diverse populations of antibodies, and these include (1) the immunization protocol such as the type of antigen, dose, route of administration, use of adjuvants, number of injections; and (2) host factors such as hormonal influences, genetic constitution, ontogenic state of the animal and phylogenetic development of the species. The purpose of the present study was to follow the changes in the antibody population during the immune response in chickens injected with dinitrophenylated-bovine gamma globulin (DNP-BGG). The population was identified in terms of the classes and the affinities of the antibodies that comprised it. Antibody classes depend on the amino acid sequence of the constant region of the heavy chain whereas affinities reflect the variable region. Antibodies with different affinities have distinct variable region amino acid sequences but two antibodies with the same affinity may still differ. Knowledge of antibody affinity also may aid in understanding the biological activities of antibodies. For example, high affinity antibody enhances

the efficiency of anaphylactic reactions (Siskind & Eisen 1965), complement fixation (Fauci et al 1970), viral neutralization (Jerne & Avegno 1958) and immune elimination (Alpers et al 1972). Steward (1974) has suggested that some chronic immune complex diseases could be due to a deficiency in the production of high affinity antibody thus allowing for the presence of complexes composed of antigen and low affinity antibody remaining in the circulation.

Some factors which influence the immune response were investigated including the dose of antigen, the number of injections and the interval between injections. In particular, since chickens differ from most mammals in producing relatively high levels of antibody within a week following a single intravenous injection of soluble antigens in saline (Wolfe 1942; Wolfe & Dilks 1946, 1948), chickens might serve as useful models in which to evaluate the role of adjuvants on the immune response. The immune response of chickens injected with Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) was characterized and compared with that obtained from chickens given intravenous injections of antigen in saline.

II. LITERATURE REVIEW

Because of the limited information available concerning the variations in the antibody population in non-mammalian vertebrates, this review will focus primarily on studies done in mammals. The first part contains a description of some fundamental terms and concepts and explanations of techniques relevant to the present study. This is followed by a discussion of the changes in the affinities and in the classes of antibodies in mammals, in chickens and in other non-mammalian vertebrates.

A. Definitions, concepts and methodology

1. Affinity

The reaction between an antibody combining site and its hapten can be represented as:

$$Ab + H \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AbH \qquad (1)$$

where Ab is the binding site, H is the hapten, AbH is the antibody-hapten complex and k_a and k_d are the rate constants for association and dissociation, respectively. The equilibrium constant (also termed the affinity constant, association constant, or intrinsic binding constant), K, describes the stability of the AbH complex and is expressed as the ratio of the rate constants:

$$K = k_a/k_d = [AbH]/[Ab][H] \qquad (2)$$

where the brackets refer to the molar concentration at equilibrium of bound hapten (AbH), free antibody sites (Ab) and free hapten (H). Since antibodies are usually heterogeneous with respect to affinity (see section II, A, 5), an average affinity value, designated K_o, is defined as the reciprocal of the free hapten concentration where half the binding sites are bound. From equation (2) it is apparent that differences in K_o could be due to changes in either the rate constant of association or dissociation or both. Direct measurements of these constants established that the equilibrium constant is governed primarily by the rate of dissociation since the rates of association differ only slightly even for antibody preparations with a large difference in K_o (Froese 1968; Hornick & Karush 1969, 1972). The binding of antibody with hapten also can be expressed in terms of the standard free energy change ($-\Delta F^\circ$) that accompanies the reaction according to the equation:

$$-\Delta F^\circ = RT \ln K_o \qquad (3)$$

where R is the universal gas constant, and T is the absolute temperature.

Several methods have been employed to measure antibody affinity. Equilibrium dialysis (Marrack & Smith 1932; Eisen & Karush 1949) is the most direct of the techniques since absolute values for the reaction can be determined. All other methods are compared to it. Some procedures depend on changes in the spectral property of either the antibody or hapten in bound form; for example, fluorescence quenching (Velick et al 1960), fluorescence enhancement (Parker et al 1967) and fluorescence polarization (Dandliker et al 1964). These techniques,

however, are based on some simplifying assumptions. The free hapten concentration is measured indirectly and changes in spectral properties due to antibody heterogeneity may influence the binding property independent of the affinity constant. The Farr technique as modified by Stupp et al (1969) is used widely because of its rapidity and its applicability to small amounts of antibody that do not require specific purification. The method consists of separating bound from free hapten by precipitation of the antibody-hapten complex with 50% saturated ammonium sulphate. Bound hapten is the difference between the total hapten added and the hapten found in the supernatant after precipitation. When compared with equilibrium dialysis, Stupp et al (1969) found that the results obtained from the Farr Method tended to give lower K_o values. Werblin et al (1973b) observed that the Farr technique fails to detect low affinity antibody in some heterogeneous populations so that K_o values may be higher than that obtained with equilibrium dialysis. The reasons for these discrepancies are not known.

2. Avidity

The equilibrium constant, K, is an intrinsic property of the antibody molecule and is independent of the valence of the antibody and of the antigen; but it is determined only by using a monovalent hapten. Antibody molecules and most antigens, however, are multivalent. The multivalency of the antibody and antigen results in a considerable enhancement of the actual binding, termed the "avidity" or "functional affinity" of the antibody. Hornick & Karush (1969, 1972) established the following relationships between the intrinsic and functional

affinity: (1) bivalent 7S antibody had a functional affinity (measured with a multivalent antigen) 10^2 times higher than its monovalent 3.5S fragment; (2) bivalent antibody had a functional affinity 10^3 times higher than its intrinsic affinity (measured with a monovalent hapten); (3) IgM preparations showed approximately 10^6 times greater functional affinity than intrinsic affinity. The enhancement apparently is due to the fact that multiple bond formation greatly decreases the rate of dissociation when compared with binding at a single site. Crothers and Metzger (1972) formulated a general theory for the determination of the increase in binding of multivalent antibodies to multideterminant antigens. They point out that at least three factors must be considered: the intrinsic affinity, the number of sites on each of the reactants, and the topological relationship of the antibody site to its determinant.

Antibody avidity generally is evaluated in relative terms. For example, Talmage and Maurer (1953) measured the dissociation of antigen-antibody precipitates by addition of excess antigen. Others have determined the tendency of antigen-antibody complexes to dissociate upon dilution (Glenny & Barr 1932; Jerne & Avegno 1956). In these cases, antibody of low avidity dissociates more easily than antibody of high avidity. The Farr technique is a commonly used method. The ratio of antibody binding capacity obtained using a low concentration of antigen is compared with that obtained using a high antigen concentration. Antibody of low avidity requires larger amounts of antigen for appreciable binding whereas antibody of high avidity may bind equally well with either concentration of antigen. The use of viruses allows more direct measurements. For example, Hornick and Karush (1972) measured the

binding of anti-hapten antibody to hapten-coupled phage in two ways: (1) directly from neutralization curves after equilibrium is reached; and (2) from kinetic measurement by determining the rate constant of association from neutralization curves and the rate constant of dissociation by the addition of free hapten causing reactivation of the phage.

3. Specificity

The binding strength of an antibody is directly proportional to the number of noncovalent interactions that the antibody can form with its determinant. Since these interactions are highly dependent on the distance between the reactants, the antibody affinity is sensitive to the "closeness of fit." For this reason an antibody generally will bind more strongly with its homologous determinant than with a heterologous one. The difference between the affinity of two similar structures is a measure of the antibody's "specificity." An antibody is highly specific if its affinity for the homologous structure is much greater than its affinity for a similar but non-identical one. In an operational sense, high affinity antibodies have low specificity because the affinity for the homologous determinant is very high; therefore reactions with heterologous determinants, although of lower affinity, are detected. Antibodies with low affinity, however, combine measurably only with their homologous antigens; thus they are considered highly specific.

Two commonly used methods for assessing the specificity of antibodies are inhibition reactions, introduced by Landsteiner (1920), and fluorescence quenching (Velick et al 1960). In the first technique,

the extent by which different haptens inhibit the precipitation of antibody with the homologous antigen is determined. In the second method, the differing abilities of haptens to quench the fluorescence of specifically purified antibody are measured.

4. Measurement of antibody binding at the cellular level

Several methods have been developed in an attempt to determine the binding properties of secreted antibody released from antibody-forming cells or of antibodies that serve as cell receptors within the cell membrane. Steiner and Eisen (1967a) devised the earliest technique. The precipitation by antigen of trace amounts of labeled antibody secreted from lymph node cells in tissue culture during a 4-hour period was measured in the presence of large amounts of an unlabeled reference antibody. Antibodies of low avidity did not precipitate as well as high-avidity antibodies when compared with a reference antibody of intermediate avidity. Presently, however, the most common technique for describing the characteristics of secreted antibody is by determining the amount of free antigen required to inhibit antibody plaque formation (Segre et al 1969; Yamada et al 1970; Andersson 1970). The method is based on the assumption that cells secreting antibody of high avidity are prevented from forming plaques with a lower concentration of free antigen than low avidity antibodies. Generally the results are recorded as that concentration of antigen capable of inhibiting 50% ($I_{50\%}$) of the plaque forming cells (pfc's). Because of its popularity this method is examined in some detail. Discussion is limited to anti-hapten systems in which hapten-coupled sheep erythrocytes

are used as the indicator and free hapten is the inhibitor since this system is less complex than those using multi-determinant antigens as inhibitors. The chief advantages of the assay are that it is rapid and simple to perform and that it is very sensitive and, therefore, it can be used before circulating antibodies are detected. In addition, antibody is measured directly from the secreting cell before possible selective removal of circulating antigen-antibody complexes can occur. Some underlying complications which exist are as follows: (1) When comparing two samples, the amount of antibody released from their pfc's may not be the same. If the amount of antibody secreted or the rate of secretion is greater for one preparation, more hapten will be required for inhibition even though the affinities are the same. (2) The experimental conditions should be carefully controlled. For example, erythrocytes substituted with high amounts of hapten increase the plaque number and the plaque size (Pasanen & Makela 1969) and would require more hapten for inhibition than erythrocytes with low amounts of hapten. (3) Comparisons should be made only between antibodies of the same class. The greater valence of IgM antibody than that of IgG antibody affects the amount of inhibitor required independent of the K_o. Many investigators assume that the direct plaques are due only to IgM-secreting cells. The presence of direct IgG plaques, however, has been suggested (Wortis et al 1969; Pasanen & Makela 1969; Pasanen 1971; Nossal et al 1971a, 1971b), although most probably few in number. When indirect plaques, i.e., plaques developed by facilitating serum (anti-γ), are assessed the plate usually contains both direct and indirect plaques. Since only indirect pfc's are desired for inhibition

studies, direct pfc's must be prevented from forming. Concanavalin A (Nordin et al 1969) may be used which interferes selectively with IgM lysis but not IgG. Also, some facilitating sera at appropriate dilutions inhibit direct while retaining the ability to enhance indirect pfc's (Dresser & Wortis 1967). A problem also exists in that pfc's to type III pneumococcal polysaccharide (Baker & Stashak 1969) apparently elicit two types of IgM antibodies: one forms direct pfc's; the other is detected only with anti-μ sera. (4) The whole population of antibodies may not be studied since only spleen or lymph node cells are examined. Preferential local synthesis of a particular kind of immunoglobulin might occur. (5) Finally, theoretical problems are inherent in this assay. It is doubtful that under the experimental conditions used, an accurate relative equilibrium constant can be determined. Attempts have been made to estimate the binding ability of cell surface receptors. Davie and Paul (1972a) developed 3 methods. The first procedure consisted of incubating sensitized lymph node cells with increasing concentrations of radioactively labeled antigen. After washing, the amount of antigen adsorbed onto the cells represented the concentration of bound antigen. The difference between the total added and bound was taken as the free antigen concentration. In the second method the association and dissociation rate constants were measured. The association rate was determined by incubating the cells with labeled antigen for varying times and stopping the reaction by a 20-fold excess of unlabeled antigen. The rate of dissociation was evaluated by dissociating the labeled antigen-receptor complex with unlabeled antigen. The ratio of the two rate constants was used as the

binding constant. The third method involved inhibiting 50% of the binding of labeled antigen to cells by free hapten. Many of the problems associated with the plaque inhibition technique (see above) also apply to these methods. In addition, Bell and DeLisi (1974) described other complications, most important of which was the high nonspecific antigen binding that occurred.

5. Heterogeneity in antibody affinity

Several classic studies have established that antibodies which combine to a given hapten exhibit considerable heterogeneity in affinity (Heidelberger & Kendall 1935; Landsteiner & van der Scheer 1936; Pauling et al 1944; Eisen & Karush 1949; Nisonoff & Pressman 1958; Karush 1956, 1957; Eisen & Siskind 1964). It was thought that antibody heterogeneity is due, in part, to the heterogeneity of the immunogen. When antigens with repeating determinants such as pneumococcal and streptococcal vaccines are used, antibodies of restricted heterogeneity have been obtained. But the reasons for this restricted response are not clear. Responses to these vaccines may be quite complex. In addition, antibodies against conventional antigens, on rare occasion, may be homogeneous. For example, Nisonoff et al (1967) reported finding a rabbit that produced an anti-p-azobenzoate antibody which crystallized spontaneously. Nevertheless even a single antigenic determinant might still elicit a heterogeneous response because of its ability to cross react with a variety of antibodies that serve as receptors on antibody-forming precursor cells. Many clones might be stimulated. Other reasons offered for restricted responses include genetic considerations,

the selective advantage of a given clone of cells or the selective tolerance of clones (Kimball 1972; Ghose & Karush 1973; Haber & Strosberg 1973).

A number of methods have been devised to study the heterogeneity of antibody affinity. Based on equilibrium dialysis data antibody affinities were assumed to be distributed in a Gaussian (Pauling et al 1944; Karush 1956) or a Sipsian (Nisonoff & Pressman 1958) manner. According to this assumption, K_o represents the average value obtained from a normal distribution curve. The extent of heterogeneity is evaluated by the degree of spread about the average constant, K_o. Utilizing a Gauss error function, the heterogeneity index, σ, is zero for homogeneous sites and increases as the heterogeneity increases. Using the Sips equation, the heterogeneity index, "a," is 1 for homogeneous sites and less than 1 for heterogeneous populations. Presently, the Sips plot is used (Karush 1962). Linearity indicates that the antibody sites are distributed in a normal array and the slope of the line is the heterogeneity index. However, it has been suggested that within each individual animal only a few molecular species of antibody exist (Pressman 1970). In fact extensive equilibrium dialysis data over a large range of free hapten concentration resulted in a non-linear Sips plot in some cases, which indicated that antibody sites were not distributed normally (Werblin & Siskind 1972b). A computational procedure was devised to describe non-Gaussian distributions. Various histograms depicting the possible distribution of affinities were generated and from these, binding curves constructed. The profile was chosen that gave a binding curve which best fitted the experimental data.

Other mathematical models using less extensive data have been developed (Roholt et al 1972: Mukkar 1974).

Antibody heterogeneity also has been studied by fractional elution from immunoadsorbent columns or by fractional precipitation. Kreiter and Pressman (1964) adsorbed anti-p-azophenylarsonate antibody on an immunoadsorbent column and eluted with different concentrations of benzenearsonate. A 12-fold difference in binding constants was obtained for the various fractions. Eisen and Siskind (1964) separated anti-DNP antibodies from individual rabbits by successive additions of DNP-BGG. Variations as great as 10^4 in binding constants were obtained from an individual rabbit. Similar studies utilizing cross-reacting haptens were performed by others (Kitagawa et al 1965a, b, c; Cheng & Talmage 1966; Hoffman et al 1971; Farah & Awdeh 1972).

The most recent technique is preparative isoelectric focusing (Freedman & Painter 1971). Hoffman et al (1972) focused specifically purified antibodies against p-azobenzoate from individual rabbits. One of the preparations resolved into 12 peaks. The relative binding constants to a variety of cross-reacting ligands were determined for some of the fractions. The cathodal-migrating fractions had binding properties in common with each other and different from the anodal-migrating fractions. Ghose and Karush (1973) examined the isoelectric profiles of specifically purified anti-lactose antibody from individual rabbits. In well isolated fractions, the antibodies were homogeneous in affinity ("a" = 1), whereas the whole antibody before focusing gave an "a" value of 0.7. Isolated fractions were homogeneous by structural characteristics such as light chain spectra, amino-terminal sequence

and allotype distribution (Freedman & Painter 1971). Thus, by both functional and structural criteria the isoelectric point appears to be a marker for each clone of antibody forming cells.

At the cellular level, binding heterogeneity is determined by the ratio of hapten concentration giving 80% inhibition of pfc's to that amount giving 30% inhibition (Davie & Paul1972b). Low values indicate restricted heterogeneity while high values designate greater heterogeneity. Avidity histograms have been constructed based on the number of plaques inhibited by 10-fold changes in free hapten concentration. Heterogeneous population is inhibited by a wide range of hapten concentrations whereas homogeneous antibody is inhibited at one hapten concentration.

B. Variations in the antibody population during the immune response in mammals

1. Changes in affinity and avidity

In 1951, Jerne published an extensive treatise examining the differences in the binding properties of antisera against diptheria toxin (Jerne 1951). He noted that sera obtained from later bleedings or after a second injection neutralized the toxin more efficiently than sera acquired early after immunization. Since then, avidity increases have been reported for anti-viral (Jerne & Avegno 1956; Svehag 1965; Finkelstein & Uhr 1966; Krueger 1970; Sarvas & Makela 1970) and other anti-protein (Talmage & Maurer 1953; Farr 1958; Grey 1964; Rubin 1971) antibodies present in the circulation. The use of anti-hapten antibodies, however, provided direct evidence that this increase in binding

was due to the increase in the average affinity constant (Eisen & Siskind 1964). This has been confirmed utilizing different haptens in many mammalian species: in rabbits producing antibodies against an amino dye (Fujio & Karush 1966), a DNP-tetrapeptide (Parker et al 1966), poly D-alanyl and penicilloyl groups (Haimovich 1969) or pneumococcal type III polysaccharide (Kimball 1972); in guinea pigs immunized against DNP or trinitrophenyl determinants (Goidl et al 1968; Little & Counts 1969; Harel et al 1970; Davie & Paul 1972b); in a horse producing antibody against p-aminophenyl-β-lactoside (Klinman et al 1966); and in rats producing anti-DNP antibody (Feldbush & Gowans 1971; Larralde & Janof 1972).

The increase in affinity during the immune response has been referred to as "maturation of the immune response" (Siskind & Benacerraf 1969). The current mechanism thought to be responsible for "maturation" is based on the clonal selection theory of Burnet (1959). In its updated form, this theory assumes that antibody forming precursor cells possess antibody receptors, the "B-cells" (bone-marrow or bursal-derived cells), before antigen contact. Antigen stimulates those cells with appropriate receptors causing them to differentiate and to proliferate, and to secrete antibody identical to that which served as the receptor. During the immune response, a selective pressure is exerted, in part, by a limiting antigen concentration. When the antigen concentration is low, cells with high binding receptors might capture antigen preferentially, proliferate and secrete high affinity antibody; cells with receptors of low affinity may persist but they are not preferentially stimulated. Thus, early in the immune response when

the antigen concentration is high, both low and high-binding cells might be stimulated. As the antigen level falls, only high-binding cells will react with the antigen. The net effect is a sequential rise of antibody affinity. This theory, termed "antigen-driven selection" has adequately explained some observations but most probably is oversimplified and requires modification.

Based on early studies by Siskind et al (1968) and by Goidl et al (1968), it was believed that injection of animals with very high or very low doses of antigen interfered with the maturation process. They thought that very high doses induced a state of partial tolerance, since less antibody was produced at the time of measuring, and that tolerance more readily affects high binding cells. This results in the production of decreased amounts of antibody with low affinity. Low affinity antibodies also were produced when suboptimal doses were administered. This was explained on the basis that the low- and average-binding cells outnumber the high binding cells. On a purely chance basis, the antigen will more likely contact low or average-binding cells when its concentration is very high. The antibody affinity will thus remain low. In these studies, however, the immunogen was incorporated into FCA which serves as a depot for the slow release of antigen. The induction of tolerance by the antigen under this condition is less likely. Adjuvants also tend to make even low doses of antigen immunogenic. More extensive work by Werblin et al (1973b) offered another explanation for the dose dependency. Their results are presented in Table II. Rabbits were given a single injection of DNP-BGG in FCA. The affinity of the antibodies obtained at various times for a 1- to 2-year period were

TABLE II

BINDING PROPERTIES[a] of 7S ANTI-DNP ANTIBODY FROM RABBITS IMMUNIZED WITH DNP-BGG IN FREUND'S COMPLETE ADJUVANT[b]

Rabbit No.	Antigen (mg)	Days after immunization	$-\Delta F_1^\circ$ (kcal/mole)	a_1	$-\Delta F_2^\circ$ (kcal/mole)	a_2
1374	.05	21	7.58	0.46	7.40	0.43
		42	10.03	0.76	9.72	0.64
		90	10.16	0.72	9.39	0.48
		180	10.45	0.93	9.41	0.62
		360	10.28	0.94	8.98	0.58
1375	.05	21	8.54	0.60	8.05	0.50
		42	10.45	0.76	10.08	0.63
		90	10.44	0.75	9.69	0.57
		180	10.97	0.97	10.10	0.64
		360	10.85	1.01	9.58	0.58
1352	0.5	11	8.19	0.68	8.19	0.68
		21	9.35	0.77	8.60	0.56
		42	10.15	0.73	9.41	0.52
		90	10.11	0.83	8.65	0.51
		180	8.05	0.38	8.21	0.41
1354	0.5	11	6.79	0.71	6.95	0.74
		21	9.19	0.91	8.44	0.70
		42	9.61	0.65	8.90	0.50
		90	10.48	0.72	9.71	0.49
		360	8.59	0.39	8.28	0.35
1361	5	7	6.64	0.76	6.79	0.79
		11	7.22	0.66	7.40	0.70
		21	8.52	0.78	8.09	0.67
		42	9.54	0.67	9.20	0.58
		90	10.83	0.87	10.10	0.64
		180	11.12	1.02	10.08	0.61
		360	10.52	0.59	9.24	0.37
1365	5	7	6.54	0.79	6.63	0.80
		11	7.56	0 70	7.62	0.72
		21	9.01	0.77	8.70	0.70
		42	10.50	0.74	9.65	0.48
		90	11.56	1.12	10.53	0.62
		180	11.61	1.14	10.85	0.54
		360	11.21	1.03	10.82	0.49
1382	50	7	7.08	0.79	7.12	0.80
		21	6.74	0.62	6.97	0.65
		42	8.68	0.56	8.55	0.54
		90	10.01	0.71	9.32	0.56
		180	11.05	0.94	10.25	0.63
		360	11.20	1.05	10.23	0.62
		720	11.17	1.02	10.40	0.48
1383	50	7	7.74	0.92	7.69	0.91
		28	9.21	0.94	9.01	0.86
		42	9.93	0.78	9.36	0.62
		90	8.12	0.44	7.31	0.37
		180	11.24	1.01	10.71	0.76

[a] $-\Delta F_1^\circ$ is the negative free energy change calculated from the Sips plot using the third of the total binding sites with the highest affinity; "a_1" is the heterogeneity index calculated from the slope of the Sips plot using a third of the sites; $-\Delta F_2^\circ$ and "a_2" are calculated using all of the sites.

[b] Table modified from Werblin et al (1973).

measured by detailed equilibrium dialysis studies. The dose of antigen appeared to affect the rate in which antibodies increased in affinity but not necessarily the absolute amount and affinity. Rabbits immunized with 0.05 mg in FCA elicited antibodies which reached maximum affinity by 42 days whereas the maximum value for rabbits immunized with 50 mg was not achieved until about 180 days. Thus the "maturation" process is characterized as a dose-dependent progressive increase in antibody affinity during the immune response. Also, a second phase which occurred very late in the immune response was noted in some rabbits in which there was a decrease in the average affinity. This was interpreted to be due to a loss of high affinity antibodies.

Preferential combination of high affinity circulating antibody with residual antigen also might explain temporal increases in K_o of antibody. These complexes would be eliminated rapidly from the blood stream (Jerne 1951). Perhaps early after immunization considerable amounts of the immunogen are present and complexes composed of the antigen and high affinity antibody would form. Only low affinity antibody would be found free in the serum. Later, when the antigen concentration decreases, high affinity antibody would be detected in the circulation. However, direct evidence excluding this possibility as the sole reason for the changes in antibody affinity has been obtained. Steiner and Eisen (1967a, b) removed lymph node cells from rabbits at various times after immunization. The relative avidities of the anti-DNP antibodies synthesized in tissue culture, i.e., free from residual antigen in the circulation were found to increase during the immune response. More recently, studies of antibodies secreted

from pfc's confirmed these results in mice (Andersson 1970; Bullock & Rittenberg 1970; Pasanen 1971; Miller & Segre 1972; Huchet & Feldmann 1973; Moller et al 1973; Claflin et al 1973; Smith et al 1974) and in guinea pigs (Davie & Paul 1972b). Measurements of antigen binding B-cells (Davie & Paul 1972b) by the methods described above (see section II, A, 4) showed an increase in binding when determined during a 30-day period. Avidity studies using hapten inhibition of rosette forming B-cells, purified by treatment of spleen cells with anti-θ serum, gave similar results (Moller et al 1973; Smith et al 1974). Nevertheless, circulating antibody may regulate the concentration of antibody produced (Bystryn et al 1970) and affect the affinity of the antibody produced in the presence of passive antibody (Walker & Siskind 1968).

Also, the affinity of the antibody produced during the secondary response has been studied. The cells responsible for the secondary response probably reflect the population of cells present immediately before boosting. Jerne (1951) found that antibodies produced after a second stimulation showed an immediate rise in avidity. Fujio and Karush (1966) noted that, as the interval between two injections of antigen was lengthened (from 2 weeks to 10 weeks), the affinity of antibodies isolated 1 week after boosting increased. In a detailed study, Steiner and Eisen (1967c) found that large amounts of high affinity antibodies were synthesized within 3 days from lymph node cells obtained from rabbits given two injections of antigen. Paul et al (1967) compared the affinity of antibodies just prior to and immediately after boosting. They found that the affinity of antibodies from animals boosted intravenously at 2 months after priming was lower than the

affinity of antibodies isolated 5 months after priming. When they compared the affinities of antibodies before and after boosting only slight differences were observed. Bullock and Rittenburg (1970) confirmed these findings using a secondary response initiated in vitro. Mouse spleen cells were challenged in vitro at different times after in vivo priming with bentonite-adsorbed trinitrophenylated keyhole limpet hemocyanin. When a long interval between priming and challenge was allowed, cells required less antigen in culture to induce the formation of pfc's than for a short interval. This indicated the presence of high binding antibody-forming precursor cells. Feldbush and Gowans (1971) confirmed the above findings and furnished additional evidence that selection of high affinity cells was antigen dependent. They reasoned that if sensitized lymphocytes were allowed to reside in an animal without the presence of the immunogen, the affinity of the antibody should not increase. Thoracic duct lymphoctes from inbred rats immunized with DNP-BGG were transferred at various times into irradiated syngeneic recipients. For controls, adoptive recipients were given cells from donors immunized 2 or 8 weeks earlier and challenged immediately. In agreement with the above studies, the affinity of the antibodies, as measured by equilibrium dialysis, was higher, although only moderately so, in those animals given cells sensitized 8 weeks earlier than those given cells primed 2 weeks earlier. For the test, recipients were given cells from donors immunized 2 weeks earlier. These cells were allowed to reside in the antigen-free recipient for 6 more weeks and then challenged. The antibody produced was of low affinity. Apparently antigen must be present for the

development of high affinity antibody.

Thus far the discussion has been limited to the binding properties of IgG antibodies. Studies with IgM antibody have been plagued with difficulties. Three controversies have arisen: (1) the problem of the valence of IgM antibody; (2) the comparison between IgM and IgG affinity, and (3) the question concerning the maturation of IgM affinity. In regard to the valence, intact IgM antibodies against high molecular weight antigens have shown an apparent valence of 5 instead of the expected value of 10 based on structural considerations (Schrohenloher & Barry 1968; Stone & Metzger 1968; Chavin & Franklin 1969; Lindqvist & Bauer 1969). This apparent valence can be attributed most easily to steric factors (Metzger 1970), i.e., the presence of the antigen at one site prevents the binding of another antigen molecule at a neighboring site. For this reason, Edberg et al (1972) examined the valence of anti-dextran IgM antibodies as a function of the size of the dextran molecule used for measuring. The valence of IgM varied between 10 for isomaltose (molecular weight 342) to 2.3 for a dextran of molecular weight 2×10^6. It appears that steric considerations adequately account for published discrepancies. Binding studies with low molecular weight ligands, however, in which steric factors are negligible have resulted in conflicting opinions. Onoue et al (1968) and Kishimoto and Onoue (1971) suggested that each IgM molecule possesses 5 high affinity sites and 5 low affinity sites based on the shape of the binding curve. Others have noted that intact IgM antibodies against haptens consist of either 5 sites (Voss & Eisen 1968; Onoue et al 1965; Clem & Small 1968; Voss et al 1969; Russel & Voss 1970; Mukkur 1972) or 5 strong and 5 weak

sites (Oriol et al 1971). Other studies using anti-hapten antibodies from lower vertebrates revealed binding curves displaying extreme heterogeneity and valences varying from 1 to 8 (Clem & Small 1970; Voss & Sigel 1972). Ashman and Metzger (1969), studying a monoclonal Waldenstrom's macro-globulin that bound nitrophenyl derivatives, gave unambiguous evidence that each IgM molecule forms 10 binding sites of equal affinity. They suggested that the dichotomy of binding sites observed by others was due to the heterogeneity of the antibody population. Metzger believes (1970) that extrapolations from Scatchard plots at very high hapten concentration may be quite arbitrary for heterogeneous antibody preparations. In addition, anti-polysaccharide antibodies clearly have a valence of 10 (Merler et al 1968; Edberg et al 1972; Kim & Karush 1973).

Because of the interest generated from attempts to understand the "IgM to IgG switch" (see section II, B, 3) several studies consisted of comparisons between the intrinsic affinity of IgM and IgG antibodies from the same serum sample. Some investigators have found a similar affinity (Onoue 1968; Jaton et al 1967; Atsumi et al 1968), whereas others reported that IgG had a greater affinity than IgM antibody (Voss & Eisen 1968; Makela et al 1970; Kim & Karush 1973). There are several reasons for these conflicting results. In some cases, comparisons between IgM and IgG affinities were made with serum pools rather than with serum from an individual animal at a given time. When specific purification was used, preferential loss of a population of antibodies might have occurred. Finally, the type of antigen, the method of immunization, the time of sampling and the species of animal used all might influence the results.

Numerous investigators also have attempted to describe possible maturation of IgM binding during the immune response. Table III summarizes these efforts. A general statement at this time is difficult to make. The most recent studies favor the possibility that maturation of IgM affinity may be completed very early during the immune response. But it is clear that systematic studies, using unambiguous affinity measurements are required before a definitive conclusion on the existence or on the rate of IgM maturation can be reached.

Although changes in the binding properties of the products of the B-cells have been characterized, properties of the T-cells (thymus-derived lymphocytes) are less well known. The T-cells are responsible for cell-mediated immune reactions (delayed-type hypersensitivity, transplantation reactions), and helper function in antibody formation, but T-cells do not secrete humoral antibodies. Unfortunately, the nature of the T-cell receptors is unknown.

Arguing for the presence of immunoglobulin-like molecules are those who observed that anti-immunoglobulin sera inhibit T-cell function such as graft versus host activity, mixed lymphocyte reaction, helper function or uptake of radioactively labeled antigen by T-cells (Mason & Warner 1970; Lesley et al 1971; Greaves & Hogg 1971; Riethmuller et al 1971). In addition, Marchalonis and his colleagues isolated immunoglobulin from murine (Marchalonis et al 1972) and chicken (Marchalonis et al 1974) thymocytes characterized as an 8S IgM monomer. Other investigators have failed to demonstrate inhibition of graft vs. host reactivity (Crone et al 1972) or the presence of immunoglobulin on T-cells (Unanue et al 1971; Vitetta et al 1972; Grey et al 1972).

TABLE III

BINDING CHARACTERISTICS OF "IgM" ANTIBODIES DURING THE IMMUNE RESPONSE

Immunogen	Animal	Binding Assay	Results	References
polio virus	rabbit	dissociation of complex by pH changes	two types of 19S: low avidity present by 2-3 days; high avidity present by 5 days	Svehag (1965)
ØX174 bacteriophage	guinea pig	dissociation of complex by dilution of mercaptoethanol-sensitive antibodies	increase from 4 days to 10 days	Finkelstein & Uhr (1966)
influenza virus	rabbit	equilibrium filtration of sucrose gradient fraction	no increase between 5 and 15 days during primary; slight increase after multiple injections	Webster (1968)
DNP-protein	rabbit horse	equilibrium dialysis after specific purification of DEAE-cellulose fraction	no increase	Voss & Eisen (1968)
sheep erythrocyte stromata	rabbit	dissociation of complex by ionic strength and temperature changes	slight increase between 2 and 15 weeks	Linscott (1969)
T4 bacteriophage	rabbit	dissociation of complex by dilution	peak at 8 days; after boosting, peak at 4 days	Haimovich & Sela (1969)
TNP-keyhole limpet hemocyanin in bentonite	mouse	direct pfc stimulation by different antigen concentrations	small increase	Bullock & Rittenberg (1970)
ØX174 bacteriophage	mouse rabbit	dissociation of complex by different ionic strengths of Sephadex fraction	increase	Hajek (1970)
alum precipitated 4-hydroxy-3-iodo-5-nitrophenyl acetyl chicken globulin with pertussis	rabbit	hapten inhibition of phage neutralization of Sephadex fraction	no increase during primary or after boost	Sarvas & Makela (1970)
p-azobenzene arsonate human serum albumin in FCA	rabbit	hapten inhibition of mercapto-ethanol-sensitive pfc's	no increase between 5 and 15 days	Wu & Cinader (1971)
Type III pneumococcal polysaccharide	Balb/C mouse	antigen inhibition of direct pfc's	no increase during primary or after boost	Baker et al (1971)
TNP-donkey enthrocytes	Balb/C mouse	hapten inhibition of direct pfc's	rapid increase completed by 5-7 days; no further increase after multiple injection	Claflin & Merchant (1972)
Salmonella typhimurium	rabbit	agglutination of DEAE-cellulose fraction	increase during primary and after boost	Schulkind et al (1972)
TNP-horse enthrocyte	C3HeB/FeJ mouse	hapten inhibition of direct pfc's	early peak; no dose dependency	Doria et al (1972)
p-azobenzene arsonate human protein in FCA	rabbit	hapten inhibition of direct pfc's	increase between 5 and 15 days at optimal dose; no further increase after boost	Wu & Cinader (1972)
DNP-fowl gamma globulin in FCA	CBA mouse	hapten inhibition of direct pfc's	no increase; very slight after boost	Huchet & Feldmann (1973)
2,4-dinitrophenyl-alanylglyglycyl-keyhole limpet hemocyanin in FCA	Balb/C N mouse	hapten inhibition of direct pfc's	increase completed by 9 days; no further increase after boost	Claflin & Merchant (1973)
p-azobenzene arsonate human serum albumin in FCA	rabbit	hapten inhibition of direct pfc's	increase between 5 and 15 days; increase after boost	Wu & Cinader (1973)
Streptococcus vaccine	horse	equilibrium dialysis after specific purification	no increase during multiple injections	Kim & Karush (1973)
Streptococcus vaccine	horse	equilibrium dialysis after specific purification	early maturation	Kim & Karush (1974)

Benacerraf (1974) suggested that a new class of molecules may serve as antigen receptors on T-cells. These molecules might have more limited variability than immunoglobulins, possess broader specificities, and might be coded by the immune response gene product.

Regardless of the structure and origin of the T-cell receptor, some experiments have attempted to reveal its binding properties. Moller et al (1973) obtained purified T- or B-cells from spleens by using cytotoxic antisera specific for anti-B- or anti-T-cells. The relative binding ability was estimated by the amount of free hapten required to inhibit rosette formation using haptenated erythrocytes and sensitized T- or B-cells. The B-cells exhibited a decrease in the amount of inhibitor required for 50% rosette formation during the immune response, whereas the T-cells did not change.

In contrast, other experiments have suggested that changes in the binding properties of T-cells might occur during the immune response (Paul et al 1968; Bast et al 1971). Using tritiated-thymidine incorporation, Paul et al (1968) showed that guinea pigs immunized with low doses of antigen in FCA yielded lymphocytes which were stimulated by relatively low concentrations of antigen in vitro whereas lymphocytes from guinea pigs immunized with high doses required higher concentrations of antigen for stimulation. Similarly, Bast et al (1971) found that lower concentrations of antigen were required for stimulation as the interval between immunization and cell culture was lengthened. These studies indicate that antigen binding ability of T-cells may increase during the immune response.

2. Changes in the heterogeneity of antibody affinity

Based on early studies using Sips heterogeneity indices, the population of antibody molecules appeared to increase in heterogeneity in parallel with the rise of antibody affinity (Eisen & Siskind 1964). In contrast, Kitagawa et al (1967) found that antibodies obtained later after immunization displayed a more restricted heterogeneity than antibodies isolated early after immunization based on fractional elution studies of specifically purified antibodies. Using a computational procedure to generate histograms from equilibrium dialysis data (see section II, A, 5), Werblin et al (1973b) described the distribution of antibody affinities during the immune response in greater detail. Early in the immune response antibody affinities were arranged symmetrically around a low K_o (Table II). With time, progressively more high affinity antibodies and less low affinity antibodies were produced resulting in a markedly asymmetrical histogram. The heterogeneity indices of the high affinity antibody population ("a_1") approached 1 for most of the animals indicating that there was a predominance of only a few clones of antibody forming cells late in the immune response. Regardless of the dose employed, the antibody of highest affinity that the animal was capable of producing was present 42 days after immunization. Thus, the total range of antibody affinities was detectable at this time but only a few clones predominated later in the immune response. Still later when the K_o dropped, an increase in "a_1" was found. Using an independent method, Ghose and Karush (1973) also demonstrated that maturation probably was due to a selection of clones producing antibody of high affinity. They examined the changing preparative isoelectric spectra of

specifically purified anti-lactose antibody obtained from individual rabbits given multiple intravenous injections of Streptococcus faecalis (strain N) over a 1-year period. In one rabbit, early during the year, the antibody produced was quite heterogeneous, forming 13 peaks, and the K_o was low. After further immunization large amounts of homogeneous antibody, resolving into only 2 major peaks, with a 100-fold greater K_o was found. The isoelectric pattern showed that the fraction with the highest affinity predominated in the monoclonal response. After a 12-week rest period when the antibody level had declined, further immunization resulted in antibodies with the same isoelectric pattern as that found in the early response. In addition, the data from both of these cases, i.e., from the Anti-DNP and from the anti-lactose responses, favor the interpretation that antigen-driven clonal selection rather than somatic mutation occurred during the immune response.

Davie and Paul (1972b), using hapten inhibition of pfc's, determined the degree of heterogeneity during the immune response by comparing $I_{80\%}$ to $I_{50\%}$ ratio (see section II, A, 5). They found that from 6 to 12 days after immunization there was a decrease in heterogeneity while from 12 to 30 days the degree of heterogeneity remained constant. They suggested that maturation was due to a loss of low avidity pfc's while high avidity cells remained. To further support this theory, they injected tritiated-thymidine into animals undergoing the maturation process (Davie & Paul 1973). They found that the high avidity plaques had recently divided and thus were labeled, whereas essentially none of the low avidity plaques were labeled. Similar profiles have been constructed for direct plaques in those cases where maturation was

observed (Claflin & Merchant 1972; Wu & Cinader 1973).

3. Changes in the antibody class

Many parameters affect the appearance of different antibody classes during the immune response. A troublesome problem in evaluating these factors however is one of methodology. Many of the earlier statements on the relative amounts of IgM and IgG required modification as the techniques used for detecting antibodies became more sensitive and the difficulty in distinguishing between the classes of immunoglobulins was realized. Nonetheless, antigens differ in their ability to elicit IgM or IgG antibodies (Pike 1967). Generally, the injection of soluble antigens in animals provokes a predominant IgM response early after immunization followed by the predominant synthesis of IgG antibody. Immunization with particulate antigens often results in the production of relatively higher amounts of IgM antibody although the assays used such as phage neutralization, hemagglutination, bacterial agglutination tend to exaggerate the IgM activity because of its greater functional affinity. Bacterial vaccines, at least in horses and cows, elicit considerable amounts of IgM antibody (Heidelberger & Pederson 1937; Kim & Karush 1973).

Related to the changes in the antibody class is the concept of IgM to IgG "switch" first postulated by Nossal et al (1964). For the present discussion, the term "switch" is assigned two definitions. First, a "switch" might occur during ontogeny in the development of B-lymphocytes. In this sense, a "switch" generally implies that the constant regions gene of the μ chain was suppressed and the constant

region gene of the chain became activated within a cell. At the genetic level, several kinds of evidence establish that a "switch" is possible and the 3 most important are: (1) a rare serum which contained a monoclonal $IgG_{2\kappa}$ and a monoclonal IgM_{κ}, known as "Till proteins," both of which had identical variable regions based on amino acid sequence studies. Immunofluorescence studies showed that different plasma cells synthesized IgG and IgM antibody yet the variable region was identical based on idiotypic determinants (Wang et al 1970). It is possible that this double paraprotein was produced from cells synthesizing only IgM antibodies which then switched to IgG synthesis. (2) Todd (1963) observed that variable region allotypic markers were present on both IgM and IgG antibodies. (3) Based on amino acid sequence data, V_H regions may be shared with any C_H genes (Edelman & Gall 1969). Also, during ontogeny, Cooper et al (1972) showed that 13-day chick embryo treated with anti-μ and bursectomized on hatching suppressed both IgM and IgG synthesis. If treatment with anti-μ was delayed until the time of hatching only IgM synthesis was suppressed. In chickens, the "switch" apparently occurred in the bursa before peripheralization. A second definition of "IgM to IgG switch" refers to that which might occur during the immune response. In this sense, a "switch" means that an animal produces predominantly IgM antibody early after immunization but later makes exclusively IgG antibody. It is implied that this is an antigen-driven event, i.e., antigen triggers the "switch" mechanism. Usually it is assumed that IgM to IgG switch occurs within individual cells, but only limited data are available to support this concept. Nossal et al (1971b) investigated

the incidence of IgM-IgG double producing pfc's using a sensitive plaque method. He found that out of 900 pfc's examined after immunization against sheep erythrocyte only 14 were double producers (1.5%). This does not favor large scale switch but perhaps it occurred too rapidly to detect. Pierce et al (1973) studied the effect of antiglobulin reagents on the in vitro immune response. Treatment of spleen cells with anti-μ diminished the primary IgG antibody response. The anti-sheep erythrocyte response of spleen cells from hyperimmunized mice was impaired by treatment with anti-γ but not with anti-μ. Thus, anti-μ suppressed the switch of IgM to IgG in the primary response but later when IgG memory cells were already formed, anti-μ had no effect. Indirect evidence indicate that T-cells might influence the switch from IgM to IgG antibodies during the immune response. Thymus-independent antigens (pneumococcal polysaccharide, lipolysaccharide, polyvinyl-pyrrolidon) appear to elicit antibody only of the IgM class (Katz & Benacerraf 1972). Furthermore, "non-responder" mice produce IgM antibody and not IgG antibody although they possess the genetic capability to produce IgG antibody. The "defect" in non-responder mice is believed to involve T-cells; perhaps this defect prevents "IgM to IgG switch."

C. Variations in the antibody population during the immune response in chickens

1. Changes in affinity and avidity

The avidity of antisera obtained from chickens given one intramuscular injection of human serum albumin (HSA) either in FCA, FIA or in saline was determined by French et al (1970) using the Farr technique.

Early in the immune response (10 days) all birds tested had antisera of low avidity. Later antisera from birds given either adjuvant treatment increased in avidity, whereas birds given the antigen in saline produced antibody of low avidity. By 49 days, no antibody was detected in the sera from birds given antigen in saline but further increases in binding were found in those chickens given adjuvant.

To date there have been no reported studies on the anti-hapten affinity changes during the immune response in chickens. Gallagher and Voss (1969) reported that specifically purified 7S anti-DNP antibodies obtained from birds given two intramuscular injections of DNP-BGG in FCA at a 30-day interval had a K_o of 1.7×10^6 as determined by equilibrium dialysis and fluorescence quenching and a valence of 2.

2. Changes in the heterogeneity of antibody affinity

No data are available concerning the distribution of antibody affinities during the immune response. Isoelectric focusing spectra of a specifically purified 7S anti-DNP antibody (Gallagher & Voss 1970) gave two protein peaks at isoelectric points of 5.6 for polymerized 7S Ig and 6.6 for the monomeric form similar to that obtained from normal 7S Ig. The isoelectric profile of a rabbit anti-DNP preparation appeared to be more heterogeneous than that of the chicken anti-DNP sample. Limited heterogeneity also was noted by autoradiographs of peptic digests of 7S anti-DNP antibodies (Benedict et al 1972), and acrylamide gel electrophoresis (Kubo unpublished data).

3. Changes in the antibody class

Both 17S and 7S antibodies have been detected in chickens immunized against a variety of antigens during the immune response. Early in the response, both 17S and 7S antibodies to bovine serum albumin were found in chickens immunized with the antigen intravenously and often 17S antibody was found slightly before the 7S antibody (Benedict 1962; Dreesman et al 1965). After repeated injections of antigen, mainly 7S antibody was formed. Some have suggested that the 17S response to protein antigens may be dose dependent (Ivanyi et al 1966; Valentova et al 1966, 1967). As in mammals, particulate antigens induce both 7S and 17S responses in chickens. The 17S antibody may be formed even after multiple intravenous injections of phage particles in birds (Uhr et al 1962; Rosenquist & Campbell 1964). Chickens immunized against sheep erythrocytes (Riha & Sviculis 1964) or Salmonella (Duffus & Allan 1968) also produce both 7S and 17S antibody.

The response of chickens to hapten protein conjugates has not been assessed for the different classes of antibodies synthesized. Sera obtained from birds 7 days after a single intravenous injection of large amounts of p-aminobenzoate BGG did not show detectable 17S binding by radioimmunoelectrophoresis (RIE) but IgA-like and 7S binding were found (Dreesman et al 1965).

D. Antibody binding in other non-mammalian vertebrates

An excellent review on the structural and functional properties of non-mammalian immunoglobulins has appeared recently (Kubo et al 1973). Some of the pertinent information is summarized here. Studies

examining the changes in the population of antibody binding have been done in elasmobranchs, osteichthyes, and reptiles. The elasmobranchs (examples include dogfish, lemon and nurse shark) possess a high molecular weight (HMW) and low molecular weight (LMW) Ig. Most probably they are of the same class of Ig based on antigenic studies and physicochemical properties of the heavy chain but the LMW Ig does not appear to be a precursor or breakdown product of the HMW Ig. Voss and Sigel (1972) compared the binding affinities of the 7S and 18S anti-DNP antibodies isolated from a nurse shark at 10 and 30 months after the first immunization. By 30 months, a total of 18 injections of DNP-protein in FCA were given. They found that the 7S antibody, as determined by equilibrium dialysis after specific purification, increased in affinity 100-fold while the 18S antibody did not change significantly. Different results were obtained from studies of the giant grouper (osteichthyes). They possess a HMW Ig in tetrameric form and a 6.4S Ig which appear to be distinct classes based on heavy chain differences. Scatchard plots of equilibrium dialysis data of 16S antibodies taken during the immune response revealed no increase in affinities. In fact, a pronounced decline was reported. Studies with 6.4S antibodies isolated from the same preparation obtained late after immunization also showed lower binding constants than those isolated from earlier bleedings. Grey (1963) examined the effect of dilution on the precipitation of KLH with anti-KLH in turtles. He concluded that turtle antibody was of lower avidity when compared with rabbit antibody and that no maturation occurred. More recent studies (Ambrosius et al 1972 quoted by Kubo et al 1973) showed the presence of anti-DNP antibodies

in tortoises with moderately high affinity (K_o = 3 X 10^8) late in the immune response.

Kubo et al (1973) noted that the rate of change in the antibody population during the immune response appeared to be slower in lower vertebrates than in mammals both in terms of "switching" from HMW to LMW Igs during the immune response and in the maturation of antibody affinity.

III. MATERIALS AND METHODS

Preparation on dinitrophenylated proteins. Bovine gamma globulin (BGG) and HSA (Pentex, Ill., Kankakee, Ill.) were dinitrophenylated as described by Eisen (1964). The conjugated proteins were rapidly isolated through a column of Dowex-1-Chloride (1X8-400) equilibrated in water. After dialysis in borate buffer (pH 8.2, $\mu/2 = 0.16$), the protein content was determined by micro-Kjeldahl analysis (Kabat & Mayer 1961). The hapten concentration was calculated from the optical density reading at 360 nm using 1.75×10^3 as the molar extinction coefficient. The molar ratios of DNP to protein in the heavily substituted conjugates were 56 to 61 for DNP-BGG and 39 to 44 for DNP-HSA.

Chicken antisera. Adult white Leghorn hens, weighing 1.5 to 2 kg, were used throughout this study. Animals were given intravenous injections of DNP-BGG in borate buffer or intramuscular inoculations of the antigen emulsified in FCA or FIA (Difco Laboratories, Detroit, Mich.) in a 1:1 mixture. The intramuscular injections were given in the leg muscle. The emulsion was checked to insure that no dispersion occurred when a drop was placed in water. The total volume injected in all cases was 1 ml. Chickens were bled by the jugular vein. A detailed description of the immunization and bleeding schedules is given in section IV. Hyperimmune anti-DNP antisera were obtained by giving at least 4 intramuscular injections of DNP-BGG in FCA over a 6-month period.

Rabbit antisera. Rabbits were injected 3 times at weekly intervals either with a mixture composed of 1.5 mg of 17S and 1.5 mg of 7S immunoglobulins (Ig) prepared as described by Benedict (1967a) or with 2 mg of a 17S-rich fraction obtained by Sephadex G-200 fractionation. All

preparations were emulsified in FCA and injected either intramuscularly or in the foot pads. Subsequent booster injections of either the globulin mixture or of diluted (1:2) normal chicken sera were given after 4 or more months to obtain anti-chicken globulin or anti-chicken whole serum, respectively. The rabbits were boosted and bled as needed. Antisera against chicken 7S Ig Fc were prepared in rabbits by giving at least 3 injections of 7S IgFc (Kubo & Benedict 1969) emulsified in FCA. The anti-Fc globulin showed a single band when tested by Ouchterlony analysis and by immunoelectrophoresis against chicken whole serum.

Globulin preparations. Although chickens were starved 24 hours before bleeding, considerable lipids and lipoprotein materials were found in whole serum from hens. These were removed using heparin and $MnCl_2$ (Lindqvist & Bauer 1966). The globulins were precipitated by the slow addition of Na_2SO_4 to a final concentration of 18% (w/v). After overnight dialysis against borate buffer all preparations were fractionated on a Sephadex G-200 column (2.5 X 100 cm). The 7S fractions were recycled on a second Sephadex column, except for those preparations devoid of 17S antibody binding activity. The void volume fractions (17S-rich material) contained 7S Ig even after repeated recycling as detected by Ouchterlony analysis using rabbit anti-7S Ig Fc. This probably represented small amounts of aggregated 7S Ig. To remove this residual 7S Ig, the 17S fractions were pooled and passed through a rabbit anti-7S Ig Fc Sepharose immunoadsorbent column (see below). After washing with 1 M acetic acid, the column was re-used repeatedly without loss in efficiency. The purified proteins were concentrated by negative pressure. Some preparations were tested for purity by

immunoelectrophoretic and Ouchterlony analyses at 10 mg/ml using appropriate antisera. Although multiple bands were visible indicating the presence of non-immunoglobulin proteins, the preparations were not contaminated with the undesired immunoglobulin.

Haptens. Dinitrophenyl-amino acids, N-ε-DNP-L-lysine HCl, Δ-N-DNP-L-ornithine HCl and N-DNP-ε-aminocaproic acid, and 2,4-dinitrophenol were obtained from Sigma Chemical Co., St. Louis, Mo. Tritiated-DNP-lysine, ε-DNP-L-lysine (phenyl-3,5-^{3}H) was purchased from New England Nuclear, Boston. The specific activity ranged from 3.2 to 5.6 Ci/mmole for different preparations. Since contaminating material in the ^{3}H-DNP-lysine sample alters the binding data (Werblin et al 1973a), the purity of the compound was checked periodically by the Farr method (Farr 1958; Stupp et al 1969). A 1:10 dilution of a hyperimmune anti-DNP antiserum was mixed with an equal volume of ^{3}H-DNP-lysine and refrigerated overnight. An equal volume of cold, saturated ammonium sulfate was added. The tubes were incubated at 4°C for a half hour and centrifuged at 2000 rpm for a half hour. A 0.1 ml aliquot was removed from the supernate and added to 2.0 ml of water and 5 ml of Aquasol (New England Nuclear, Boston) and counted in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., La Grange, Il.). The per cent binding was determined from the counts obtained using 1:10 normal chicken serum control. When the purity of the compound was less than 95%, the product was purified on silica gel thin layer plates (Schleicher & Schuell, Inc., Keene, N. H.) using ammonium hydroxide and n-propanol (1:2.5) as the solvent system. After chromatography, the plates were dried and placed on X-ray film (No-Screen, Eastman Kodak Co.,

Rochester, N. Y.) for 7 days. Two impurities were detected: a minor one with an R_f of 0.9 and a major one with an R_f of 0.6. The band corresponding to DNP-lysine, as determined with a known control (R_f = 0.7), was eluted from the silica gel and stored in borate buffer.

Immunoelectrophoresis and radioimmunoelectrophoresis. Immunoelectrophoresis was performed as described by Scheidegger (1955). For radioimmunoelectrophoresis (RIE) (Yagi et al 1962) rabbit anti-chicken globulin was used to develop the precipitin bands, and binding activity was demonstrated by using DNP-HSA or BGG labeled with either ^{131}I or ^{125}I according to the method of Hunter and Greenwood (1962).

Passive hemagglutination (PHA). Hemagglutinating antibody was detected by using tanned sheep erythrocytes (Stavitsky 1954) coupled with either DNP-HSA for the detection of anti-DNP antibodies or BGG for the titration of anti-BGG antibodies.

Precipitation in gel. Double diffusion in agar gel using either petri dishes or microscope slides was employed to detect precipitins (Ouchterlony, 1953). For chicken precipitating antibody, the agar contained 1.5 M NaCl (high salt).

Preparation of immunoadsorbents. Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was activated by treating with 50 to 100 mg of cyanogen bromide per ml of packed Sepharose for 10 min in the cold at pH 11 (Cuatrecasas 1970). For DNP-lysyl immunoadsorbents a low coupling of ligand was preferable to high substitution. Considerable non-specific binding of serum proteins remained firmly attached to highly substituted columns. Controlling the amount of coupling was achieved by limiting the ligand concentration. For obtaining 2 μmoles

DNP/ml of packed Sepharose, a solution of 1 X 10^{-2} M DNP-lysine in 0.1 M sodium bicarbonate buffer, pH 9.2, in a volume equal to that of the Sepharose was allowed to react with the activated Sepharose by rotating at 10 rpm at 5°C overnight. Vigorous mixing was avoided which tended to disrupt the bead form. The coupling ratio was determined by subtracting the amount eluted after washing from the total ligand added. Before use, the immunoadsorbent column was treated with a half volume of 1% BSA, followed by borate buffer and 25% acetic acid. The column was then washed extensively with buffer. The capacity of the immunoadsorbent was approximated by passing hyperimmune anti-DNP serum through a small column (0.5 ml total volume). After extensive washing, the protein eluted with 1 M acetic acid was determined by O.D. reading at 280 nm. The capacity of DNP-lysyl Sepharose at a coupling ratio of 1.9 μmoles per ml of Sepharose for hyperimmune anti-DNP antibody was greater than mg/ml immunoadsorbent. In some of the studies, DNP-lysyl Sepharose with a coupling ratio of 4 μmoles per ml was used.

For the coupling of chicken 7S Ig or of specifically purified rabbit anti-chicken 7S IgFc (see below), 10 mg of protein per ml of activated Sepharose in 0.2 M citrate buffer, pH 6.4 was used. More than 94% of the protein was coupled. The capacity of the column was determined in the same manner as was done for the DNP immunoadsorbent or, more simply, by adding known amounts of the ligand and determining saturation by monitoring the eluant for the first detection of protein. Rabbit anti-7S IgFc Sepharose retained about 2 mg 7S Ig per ml Sepharose.

Specific Purification. For the preparation of specifically purified anti-DNP antibodies, whole chicken serum was centrifuged at 10,000 rpm

for 20 min and a 1:100 dilution of 0.1 M EDTA was added. The serum was applied to an immunoadsorbent column which had a volume at least twice as great as the expected capacity. The column was washed extensively with borate buffer until the O.D. of the eluate was less than 0.02. The column was then unpacked and the contents placed above a Dowex column equilibrated in buffer. Sequential elutions were then performed. A solution of 0.1 M dinitrophenol (DNP-OH) titrated to pH 8.2 with NaOH in a volume equal to the amount of Sepharose was passed through the columns. The hapten remained firmly bound to the Dowex column, and the specifically purified sample was collected. The procedure was repeated using 0.1 M DNP-glycine and finally with 0.1 M DNP-ε-amino-caproate. To specifically purify rabbit anti-chicken 7S Ig Fc, an immunoadsorbent column containing 7S Ig was employed. Glycine-HCl buffer, pH 2.5, was used to elute the rabbit anti-Fc. The anti-Fc was dialyzed against 0.2 M sodium citrate buffer, pH 6.4, and used for coupling onto Sepharose (see above).

Equilibrium dialysis. For globulin preparations, the technique used was similar to that described by Nisonoff and Pressman (1958) and modified by Werblin and Siskind (1972). Tritiated DNP-lysine was added to appropriate concentrations of cold DNP-lysine or diluted directly in borate buffer made up to 100 or 200 ml. The larger volume was used for low hapten concentrations ($<1 \times 10^{-9}$ M). Each bottle usually contained 13 dialysis bags (0.25 inch inflated diameter, A. H. Thomas Co., Philadelphia, Pa.) which contained 5 different antibody preparations each in duplicate, duplicate samples of normal chicken 7S or 17S globulin, and borate buffer. Occasionally as many as 7 different

antibody samples were run simultaneously. Equilibrium was established by rotating the bottles at 10 rpm for at least 72 hr at 5°C. Because of the low antibody concentration in some of the globulin samples and the limited amount of material, the following procedures were followed. A test run was performed using a single hapten concentration, initially at 2×10^{-6} M, and the amount of globulin required for significant binding was determined. Usually the counts for the antibody samples were 2 or more times greater than that obtained for the normal 7S or 17S globulin. These protein concentrations were used for bottles containing initial hapten concentrations of 1×10^{-6} M to 5×10^{-6} M. For 17S globulin samples less than 10 mg/ml of globulin was required but for some 7S antibody preparations as much as 40 mg/ml of globulin was needed. Thus, only 0.2 ml of some samples per bag was employed for the part of the curve using high hapten concentrations. The remainder of the data was obtained by measuring 4 or 5 hapten concentrations at a time using lower concentrations of globulin. The minimum concentration utilized was 0.1 mg/ml of globulin. Since bag drying or volume changes alter the results significantly, the protein concentrations were determined by the Folin reaction (Lowry et al 1951) on aliquots from each bag after equilibrium was established. Standard curves for the Folin reaction were determined for 7S Ig using an extinction coefficient of 1.35 ($E_{280nm}^{1mg/ml}$) and for 17S Ig ($E_{280nm}^{1mg/ml} = 1.25$). Samples from each bag were added to either 5 ml (sample size $\leq$1 ml) or 10 ml (sample >1 ml) of Aquasol and placed in a scintillation spectrometer until 20,000 counts had accumulated.

For specifically purified samples, the concentrations of 7S antibody used ranged from 0.416 to 4.38 mg/ml and those of 17S antibody ranged from 0.215 to 3.47 mg/ml. Only high initial hapten concentrations were used from approximately 1×10^{-7} M to 5×10^{-5} M.

Calculations. Calculations of the binding data obtained on the globulin preparations were performed with the aid of an APL/360 computer program (written by Dr. Arthur Popper, Dept. of Zoology, University of Hawaii). The concentration of hapten bound antibody was standardized so that all values could be treated as if they were obtained at a globulin concentration of 10 mg/ml. The following serves as an example of the procedure employed. The concentration of hapten bound for the antibody or normal globulin was determined by subtracting the concentration of free hapten, obtained from the bag containing buffer alone, from the total hapten concentration found within each protein bag. The protein concentrations were determined from the Folin reaction. For example, if the amount of antibody globulin was 30 mg/ml, then the concentration of the hapten bound was divided by 3, whereas if the amount of antibody was 1 mg/ml, the concentration of bound hapten was multiplied by 10. Similarly, the hapten bound by normal globulin was adjusted to 10 mg/ml. The hapten bound to antibody was corrected for the non-specific binding by normal globulin and the duplicates were averaged.

The total concentration of antibody combining sites was determined from the following equation (Nisonoff & Pressman 1958):

$$\frac{1}{B} = \frac{1}{B_t} \times \frac{1}{K} + \frac{1}{C} \qquad (4)$$

where B is the amount of hapten bound, B_t is the total concentration of antibody binding sites, C is the free hapten concentration and K is the average affinity constant in the manner described by Werblin and Siskind (1972). When the data are plotted in the form 1/B versus 1/C the y-intercept is determined from linear regression analysis. This extrapolated value is $1/B_t$, where $C \rightarrow \infty$, the reciprocal of the total concentration of the antibody combining sites. The concentration of 7S antibody was calculated from B_t by using a molecular weight of 1.7×10^5 daltons (Table 1) and assuming a valence of 2. For 17S antibody, a molecular weight of 8.9×10^5 daltons (Table I) was used and a valence of 10 was assumed. These values are presented as the concentration of antibody per 10 mg of 7S globulin or the concentration of antibody per 1 mg 17S globulin. The sources of error, advantages and disadvantages of this method for determining antibody concentration are described in section V.

Antibody affinity was calculated using the Sips plot (Karush 1962; Werblin & Siskind 1972):

$$\log B/(B_t - B) = \log C + \log K_o \qquad (5)$$

and $-\Delta F^\circ$ values (standard free energy change) were determined at 5°C from equation (3)

$$-\Delta F^\circ = RT \ln K = 1.273 \log K$$

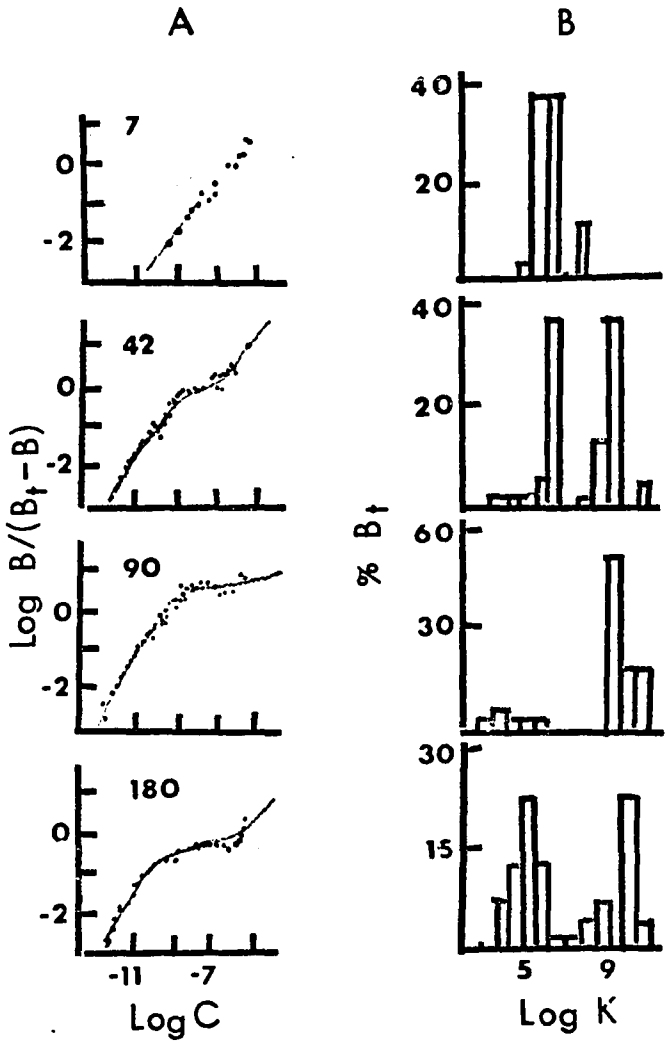
where R is the universal gas constant (1.987×10^{-3} kcal/degree/mole), T is the absolute temperature (°C + 273.15) and ln K is the natural logarithm of K (2.303 log K). The heterogeneity index "a" was determined from the slope of the regression line. Two sets of values were obtained:

(1) $-\Delta F_1^\circ$ and "a_1" were measured by considering 33% of the total sites; (2) $-\Delta F_2^\circ$ and "a_2" were calculated using essentially all of the sites. The first values are convenient for comparing different antibody populations (Werblin & Siskind 1972) and represent those sites of highest affinity. The "a_1" value determined from these points describes the heterogeneity for that selected population and would tend to be less than that represented for the whole population. The $-\Delta F_1^\circ$ may be appreciably higher than $-\Delta F_{av}^\circ$ (defined by Werblin & Siskind 1972) which appears to be a more accurate figure for the average affinity of all the binding sites. The second value, $-\Delta F_2^\circ$, seems to correspond more closely to $-\Delta F_{av}^\circ$ (Werblin et al 1973b) although this need not be so necessarily. Samples in which $-\Delta F_1^\circ$ and $-\Delta F_2^\circ$ are identical indicate that the antibodies are distributed in a normal array.

The shape of the binding curves gives information on the distribution of antibody affinities. Several curves and their corresponding histograms generated from detailed computer analyses have been published (Werblin et al 1973b). It can be determined whether selection for high affinity antibody has occurred by visual inspection of the Sips plots although subtle differences will be missed. Figure 1 shows examples of Sips plots and histograms as modified from Werblin et al (1973b). Antibodies that exhibit a normal distribution show a linear Sips plot and the $-\Delta F_1^\circ$ and $-\Delta F_2^\circ$ are similar. A shift in the distribution of antibody affinities is signified by a deviation from linearity and large differences between $-\Delta F_1^\circ$ and $-\Delta F_2^\circ$.

Equilibrium dialysis data from specifically purified samples were analyzed by the Scatchard plot according to the equation

Figure 1. Determination of the distribution of binding constants. Examples of the Sips plots (a) and the corresponding histograms (B) were taken from Werblin & Siskind (1972b). Rabbits were given a single injection of 5 mg DNP-BGG in Freund's complete adjuvant and bled on the days indicated (number in upper left corners). Note that Sips plots which are linear give histograms with a normal distribution of affinities, whereas non-linear Sips plots yield histograms that have an asymmetric distribution.


A
B
7
42
90
180
0
-2
-11
-7
Log C
Log B/(Bt-B)
40
20
60
30
15
5
9
Log K
% Bt

$$\frac{R}{C} = -RK + NK \qquad (6)$$

where R is the molar ratio of hapten bound/total antibody concentration and N is the number of binding sites (valence) per antibody molecule.

IV. RESULTS

A. Qualitative changes in 7S and 17S antibody response to DNP-BGG

1. Intravenous immunization

The immune response of chickens given intravenous injections with heavily substituted DNP-BGG was characterized. Radioimmunoelectrophoresis was used as a rapid qualitative method to detect 7S and 17S antibodies. Initially 5 chickens were studied. Three were immunized with 24 mg DNP-BGG in saline and 2 were given 8 mg of antigen in saline. Figure 2A illustrates some RIE patterns obtained from analyzing the primary antisera from chicken 1204. Table IV tabulates a relative assessment of the 17S and 7S antibody responses for all of the chickens. By 3 days after 1 intravenous injection, slight 17S anti-DNP antibody activity was observed in the sera as indicated by the weak binding of radio-labeled antigen. At 5 days, both 17S and 7S antibodies were detected but by the 22nd day binding by both antibodies was low. Little or no activity was evident by 34 days. The response was more vigorous following a second intravenous injection (Figure 2B, Table IV) using the same dose as for the primary response. Binding of antigen both in the 17S and 7S Ig arcs was apparent from 3 to 27 days. In addition 17S binding was detected by RIE for at least 136 days. Even after a third injection both 17S and 7S antibodies were produced for 106 days.

The primary response consisted of low levels of hemagglutinating of antibodies as determined by PHA (Figure 3). Two of the birds possessed titers of 10 and only 1 chicken had a titer above 80. Furthermore, no high-salt precipitating antibodies were detected by Ouchterlony analysis.

Figure 2. The synthesis of 7S and 17S anti-DNP antibodies as detected by radioimmunoelectrophoresis. Chicken 1204 was immunized with 24 mg of DNP-BGG intravenously (A) followed by a second intravenous injection of 24 mg of DNP-BGG 34 days later (B). Chicken 1252 immunized intramuscularly with 8 mg of DNP-BGG in Freund's complete adjuvant (C). Numbers refer to the days following immunization.

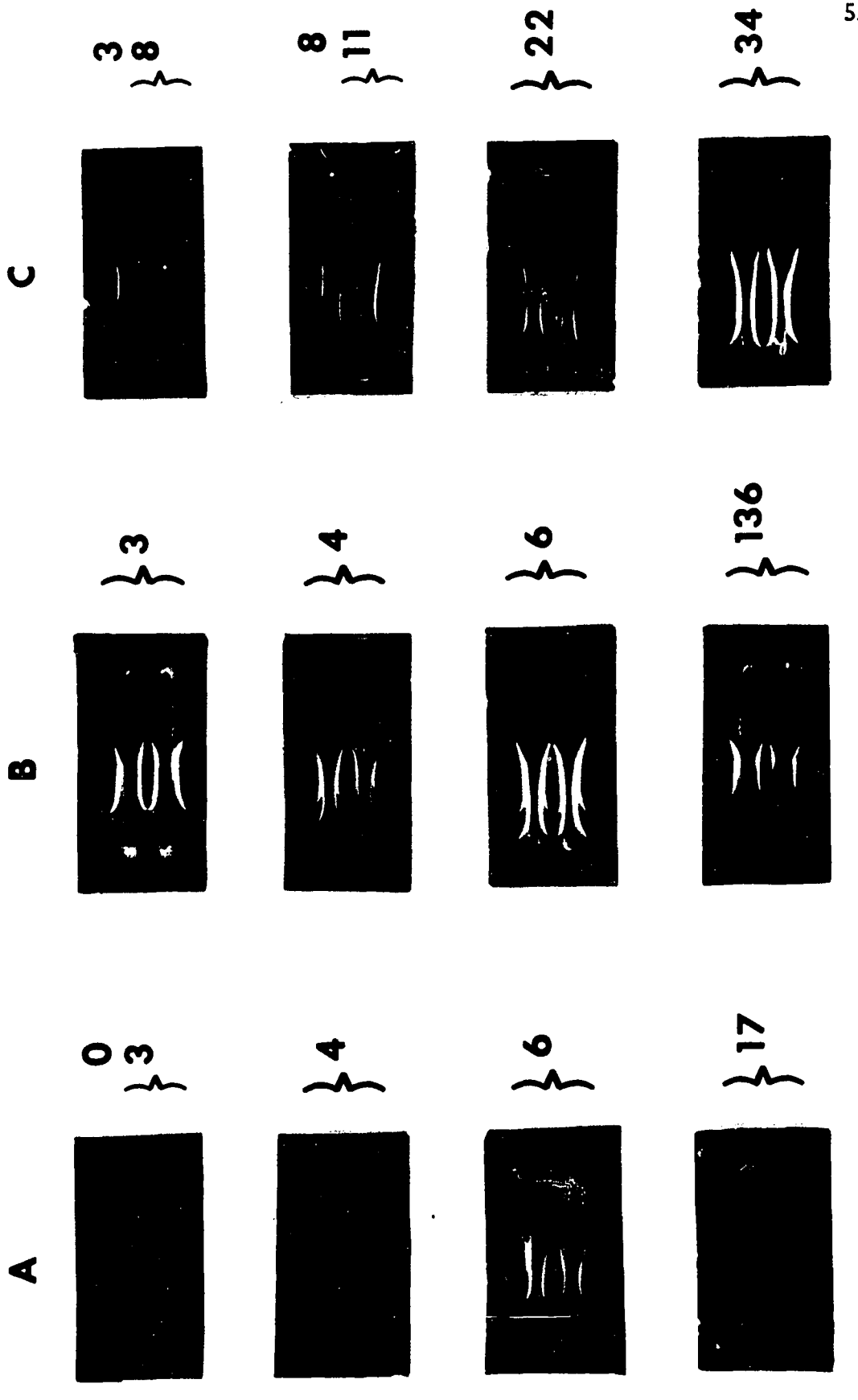

A
B
C
0
3
4
6
17
3
4
6
136
3
8
8
11
22
34

TABLE IV

RELATIVE BINDING ACTIVITY[a] OF 17S AND 7S ANTI-DNP ANTIBODIES FROM CHICKENS GIVEN INTRAVENOUS INJECTIONS OF DNP-BGG

Days following immunizations	Chicken number									
	17S antibody					7S antibody				
	1202	1203	1204	1205	1206	1202	1203	1204	1205	1206
0[b]	0	0	0	0	0	0	0	0	0	0
3	+	+	+	+	+	+	0	+	0	0
4	++	+	+	+	+	+	0	+	+	0
5	++	+	++	++	++	++	+	+	+	+
6	+	+	+	++	++	++	++	++	++	++
8	+	+	+	+	+	++	++	++	+	+
11	+	+	+	+	+	++	++	++	+	+
17	+	0	+	+	+	++	+	+	+	0
22	+	0	+	+	+	+	+	+	+	0
34	0	0	+	0	0	0	0	+	0	0
1[c]	0	0	0	+	0	+	0	0	0	0
2	+	0	+	+	+	+	0	+	0	0
3	++	++	++	++	++	++	0	+	+	+
4	++	++	++	++	++	++	++	++	+	+
5	++	++	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++	++	++	++
8	++	++	++	++	++	++	++	++	++	++
11	++	++	++	++	++	++	++	++	++	++
17	++	+	++	++	++	++	++	++	+	+
27	++	+	++	++	++	++	+	+	+	+
34	++	+	++	++	++	0	0	+	+	+
53	+	+	++	++	++	0	0	+	0	0
68	+	+	++	++	++	0	0	+	0	0
87	+	+	++	++	+	0	0	+	0	0
136	+	+	++	++	+	0	0	+	0	0
2[d]	(-)[e]	+	++	++	++	(-)	0	++	+	++
4		++	++	++	++		++	++	++	++
9		++	++	++	++		++	++	++	++
19		++		++	++		++		++	++
38		++		++	++		++		++	++
106		(-)		++	+		(-)		+	++

[a] Relative binding activity as detected by radioimmunoelectrophoresis: 0 = no binding; + = weak binding; ++ = strong binding; blanks = not done.

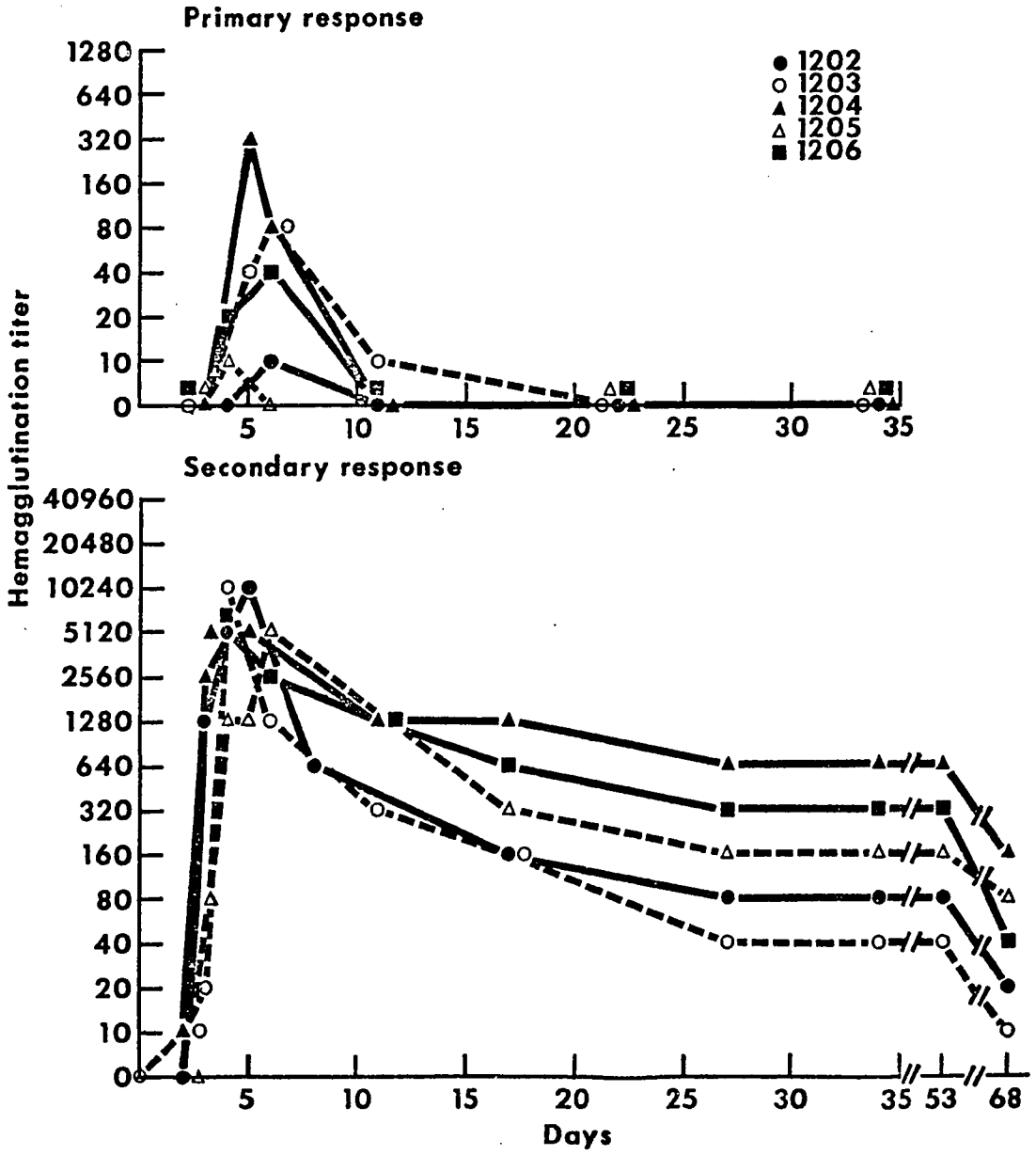
[b] First injection: chickens 1202, 1203 and 1204 were injected with 24 mg of DNP-BGG in saline; chickens 1205 and 1206 were given 8 mg of antigen.

[c] Second injection given 34 days after the first using the same doses as for the primary.

[d] Third injection given 156 days after the second using the same doses as for the primary and secondary.

[e] Chicken died.

Figure 3. The primary and secondary anti-DNP hemagglutination response of chickens given 2 intravenous injections of DNP-BGG. The dose of antigen for both injections was 24 mg for chickens 1202, 1203 and 1204 and 8 mg for chickens 1205 and 1206. The interval between injections was 34 days.


Primary response
1280
640
320
160
80
40
20
10
0
5
10
15
20
25
30
35
1202
1203
1204
1205
1206
Secondary response
Hemagglutination titer
40960
20480
10240
5120
2560
1280
640
320
160
80
40
20
10
0
5
10
15
20
25
30
35
53
68
Days

In the secondary response, the sera from all birds contained hemagglutinating titers of 5120 or higher at 6 to 8 days (Figure 3) and high-salt precipitating antibodies by 4 days after boosting.

The antibody response to the carrier protein, BGG, also was examined by RIE using ^{131}I-labeled BGG. As shown in Table V, after 1 injection of DNP-BGG, the serum of only 1 of the 5 birds (chicken 1204) bound the carrier protein and this was due to 17S antibody. Also, only chicken 1204 had appreciable but low (1:40) hemagglutinating antibody after 1 injection (Figure 4). Perhaps the high substitution of the hapten on BGG altered the protein so that only a few determinants were common to native BGG and to DNP-BGG (Ashley & Ovary 1965). In the secondary response, all birds elicited both 17S and 7S antibodies to the native BGG which may have been directed to the few common determinants. The PHA titers were low ($\leq$640) even after 2 injections except for chicken 1204 which had responded after the primary injection (Figure 4).

In these initial experiments, 8 and 24 mg of DNP-BGG induced 7S and 17S anti-DNP antibody in all birds. In order to determine the minimal dose required to elicit detectable antibody by an intravenous injection, groups of chickens were given 1 intravenous immunization of either 0.05, 0.5 or 2.0 mg of DNP-BGG in saline. The sera from all birds injected with the higher doses, 0.5 or 2.0 mg, possessed 17S binding 6 days after immunization (Table VI) although 2 of the 5 birds injected with 0.5 mg failed to produce detectable 7S antibody after one injection. With the lower dose, 0.05 mg, 3 of 5 chickens failed to respond. A second injection with the same dose as that used in the primary immunization stimulated responses in all birds that were qualitatively indistinguishable

TABLE V

RELATIVE BINDING ACTIVITY[a] OF 17S AND 7S ANTI-BGG ANTIBODIES FROM CHICKENS GIVEN INTRAVENOUS INJECTIONS OF DNP-BGG

Days following immunization	Chicken number									
	1202	1203	1204	1205	1206	1202	1203	1204	1205	1206
	17S antibody					7S antibody				
0[b]	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	+	0	0	0	0	0	0	0
6	0	0	+	0	0	0	0	0	0	0
8	0	0	++	0	0	0	0	0	0	0
11	0	0	+	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0	0	0
1[c]	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	+	0	0	+	0	0	0	++	0	0
4	+	+	++	+		+	+	++	+	
5	+	+	++	+	++	+	+	++	+	+
6	+	+	++	+	+	++	+	++	+	+
8	+	0	++	+	+	++	+	++	+	++
11	+	0	++	+	+	++	+	++	+	++
17	+	0	++	+	+	+	+	++	+	++
27	+	0	++	0	0	0	0	++	0	0
34	0	0	+		0	0	0	+	0	0
87	0	0	+	0	0	0	0	+	0	0

[a] Relative binding activity as detected by radioimmunoelectrophoresis: 0 = no binding; + = weak binding; ++ = strong binding; blanks = not done.

[b] First injection: chickens 1202, 1203 and 1204 were injected with 24 mg of DNP-BGG in saline; chickens 1205 and 1206 were given 8 mg of antigen.

[c] Second injection given 34 days after the first using the same doses as for the primary.

Figure 4. The primary and secondary anti-BGG hemagglutination response of chickens given 2 intravenous injections of DNP-BGG. The dose of antigen for both injections was 24 mg for chickens 1202, 1203 and 1204 and 8 mg for chickens 1205 and 1206. The interval between injections was 34 days.

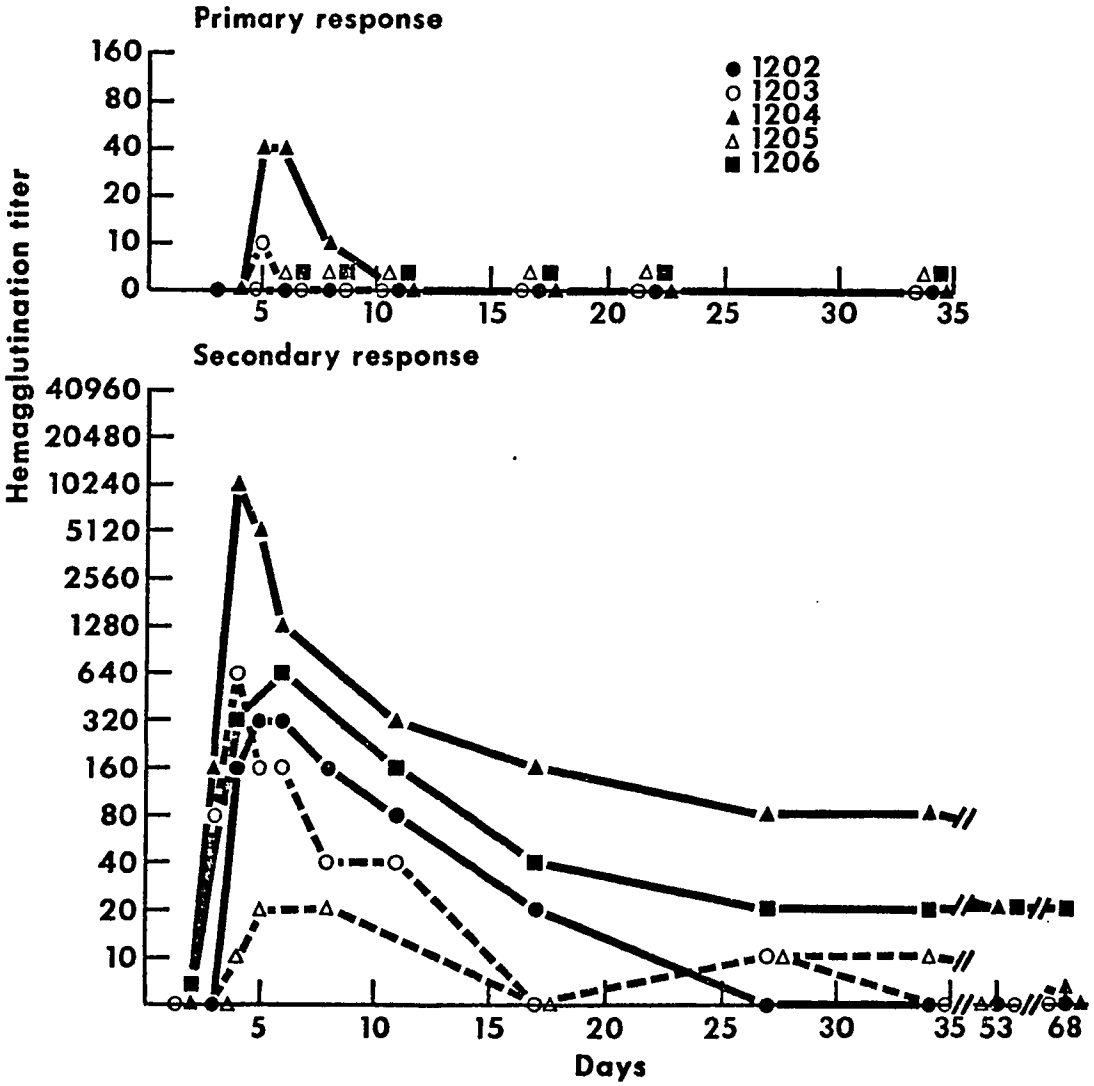

Primary response
160
80
40
20
10
0
5
10
15
20
25
30
35
● 1202
○ 1203
▲ 1204
△ 1205
■ 1206
Secondary response
Hemagglutination titer
40960
20480
10240
5120
2560
1280
640
320
160
80
40
20
10
5
10
15
20
25
30
35
53
68
Days

TABLE VI

RELATIVE BINDING ACTIVITY[a] OF 17S AND 7S ANTI-DNP ANTIBODIES FROM CHICKENS GIVEN INTRAVENOUS INJECTIONS OF VARIOUS DOSES OF DNP-BGG

Chicken number	Antigen (mg)	Days after first injection												Days after booster injection[b]											
		0		3		6		10		20		39		3		6		15		34		50		106	
		17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S
1278	2.0	+	0	+	+	++	+	++	+	++	+														
1279		0	0	+	0	++	0	++	+	++	0														
1280		0	0	+	+	++	+	++	++	+	+			++	+	++	+	++	+	++	+	+	0		
1281		0	0	+	+	++	++	++	+	++	++	+	0	++	++	++	++	++	+	++	+	++	+	++	0
1282		0	0	+	0	+	+	+	+	+	0	0	0												
1283	0.5	0	0	+	0	++	+	++	+	++	+	++	0	++	++	++	++	++	++	++	+			++	0
1284		0	0	+	0	++	0	++	0	+	0	0	0												
1285		0	0	0	0	++	0	++	0	+	0	0	0												
1286		0	0	0	0	++	+	++	0	+	0	++	0	++	+	++	++	++	++	++	+	++	+	++	0
1287		+	0	+	0	++	+	++	0	+	0	++	0												
1293	0.05	0	0	++	0	++	0	++	0	+	0	+	0	++	0	++	++			++	+	++	+		
1294		0	0	0	0	0	0	0	0	0	0	0	0	+	0	+	++	++	+	++	+	++	0	+	0
1295		0	0	0	0	0	0	0	0	0	0	0	0	+	0	++	++	++	+	++	0	++	0		
1296		+	0	+	0	+	+	+	+	+	0	+	0	++	+	++	++	++	++	++	+	++	+		
1297		0	0	0	0	0	0	0	0					+	0	++	+	++	+	+	0	0	0		

[a] Relative binding activity as detected by radioimmunoelectrophoresis: 0 = no binding; + = weak binding; ++ = strong binding; blanks = not done.

[b] Second injection given 39 days after the primary using the same dose as in the first injection.

from each other at 6 days. Synthesis of 17S antibody continued for at least 50 days even if binding was not detected after one injection.

Although these data lacked quantitation, it was concluded that one intravenous injection of DNP-BGG in saline produced fleeting 17S and 7S antibody responses and that a second injection of antigen produced a more vigorous response which led to detectable synthesis of 17S antibody for at least 4 months.

2. Intramuscular immunization with antigen emulsified in FCA

Initially 5 chickens were injected with 8 mg of DNP-BGG in FCA, a dose of antigen which had been used for the previous studies on the response to intravenous injections. Antigen binding in the 17S arc was found in 2 of the 5 birds early after immunization (Table VII). The other 2 chickens did not elicit detectable 17S antibody but they produced 7S antibody. The pre-immune sera from these 2 birds also demonstrated binding in the 7S arcs (see section V). By the 11th day, all birds showed only 7S binding activity. Chicken 1252 exhibited a "switch" from predominantly 17S synthesis on the 9th day to exclusive 7S antibody by 11 days. The radioimmunoelectrophoretic patterns obtained for various sera from this bird are shown in Figure 2C. In all cases, the 7S binding remained intense for 34 days. A second injection of antigen in FCA at this time resulted in the continued production of 7S antibody.

The PHA anti-DNP titers were low. The highest titer obtained during the primary response was 80 (Figure 5). Even after the booster injection the maximum titer was 1,280 in contrast to the maximum titer of 10,240 found in chickens immunized intravenously.

TABLE VII

RELATIVE BINDING ACTIVITY[a] OF 17S AND 7S ANTI-DNP ANTIBODIES FROM CHICKENS IMMUNIZED WITH DNP-BGG IN FREUND'S COMPLETE ADJUVANT

Days following immunizations	Chicken number									
	1250	1251	1252	1253	1254	1250	1251	1252	1253	1254
	17S antibody					7S antibody				
0[b]	0	0	0	0	0	++	0	0	+	0
3	0	+	+	0	++	++	+	0	+	0
4	0	+	+	0	++	++	+	0	+	0
5	0	+	+	0	++	++	+	0	+	0
6	0	+	+	0	++	++	++	0	+	+
8	0	0	+	0	0	++	++	+	++	++
11	0	0	0	0	0	++	++	++	++	++
18	0	0	0	0	0	++	++	++	++	++
22	0	0	0	0	0	++	++	++	++	++
34	0	0	0	0	0	++	++	++	++	++
2[c]	0	0	0	0	0	++	++	++	++	++
3	0	0	0	0	0	++	++	++	++	++
4	0	0	0	0	0	++	++	++	++	++
6	0	0	0	0	0	++	++	++	++	++
8	0	0	0	0	0	++	++	++	++	++
11	0	0	0	0	0	++	++	++	++	++
18	0	0	0	0	0	++	++	++	++	++
26	0	0	0	0	0	++	++	++	++	++
35	0	0	0	(-)[d]	0	++	++	++	(-)	++
59	0	0	0		0	++	++	++		++
89	0	(-)	0		0	++	(-)	++		++

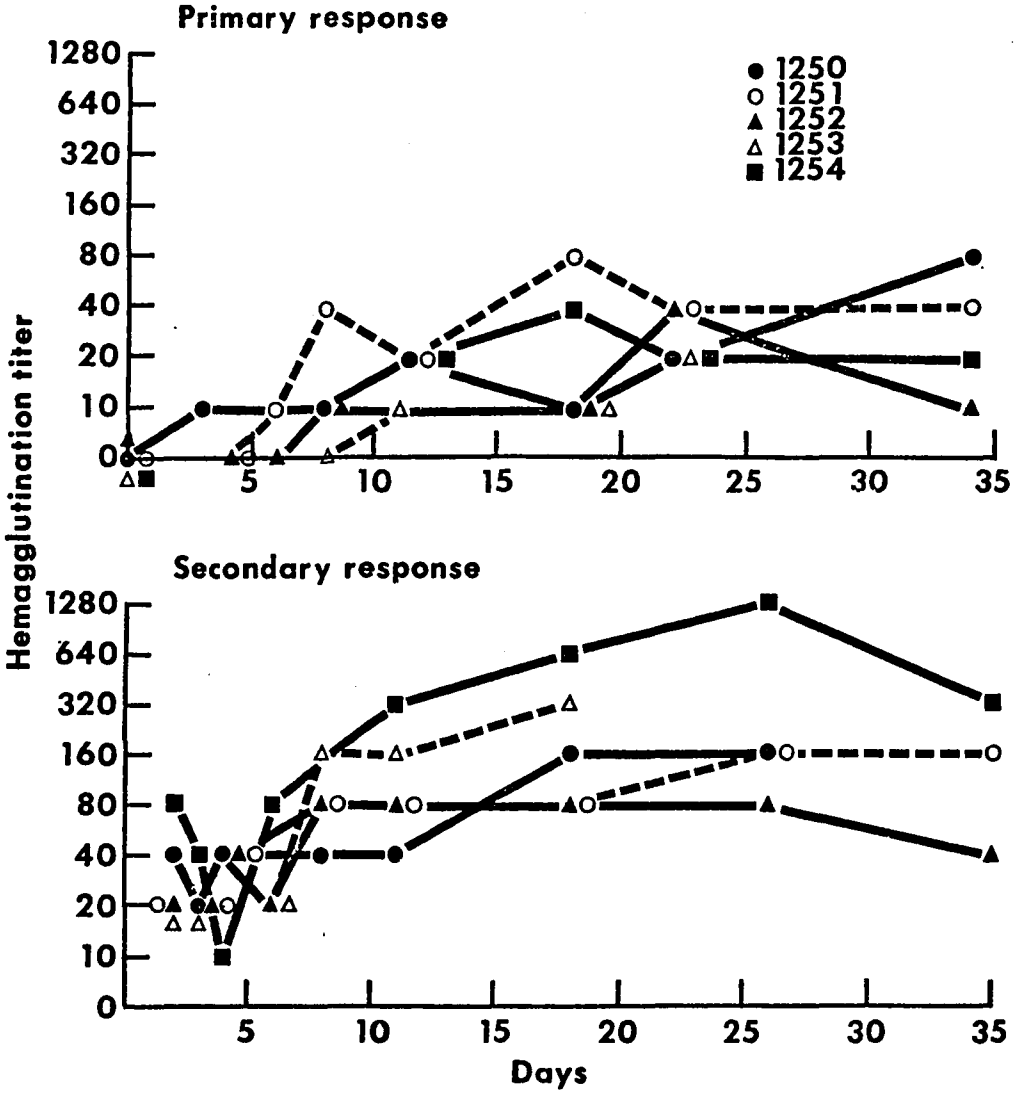
[a] Relative binding activity as detected by radioimmunoelectrophoresis: 0 = no binding; + = weak binding; ++ = strong binding; blanks = not done.

[b] First injection: chickens immunized with 8 mg of DNP-BGG in FCA.

[c] Second injection: chickens boosted with 4 mg of DNP-BGG in FCA 34 days after the primary immunization.

[d] Chicken died.

Figure 5. The primary and secondary anti-DNP hemagglutination response of chickens given 2 intramuscular injections of DNP-BGG in Freund's complete adjuvant. The doses of the first and second injections were 8 and 4 mg of antigen, respectively. The interval between injections was 34 days.


Primary response
1280
640
320
160
80
40
20
10
0
5
10
15
20
25
30
35
1250
1251
1252
1253
1254
Hemagglutination titer
Secondary response
1280
640
320
160
80
40
20
10
0
5
10
15
20
25
30
35
Days

The anti-carrier responses were studied in these birds injected with antigens in FCA (Table VIII). After one injection of DNP-BGG in FCA, all birds produced 7S antibodies with activity to native BGG, although only 3 of the 5 animals had detectable 17S antibodies. Thus, the primary anti-carrier response was more consistent than that observed in the intravenously injected birds. After boosting with DNP-BGG, chicken 1253 elicited both 7S and 17S antibodies; all other birds produced only 7S antibodies. Similar to the anti-DNP antibodies, the PHA titers for the anti-BGG antibodies were low ($\leq$1280) after 1 and 2 injections (Figure 6).

To study the effect of dose of antigen and the duration of the anti-DNP response in chickens given a single injection, groups of birds were immunized with either 0.02, 0.2, 2 or 20 mg of DNP-BGG in FCA (Table IX). Compared to chickens injected with higher doses, the formation of detectable levels of 7S antibodies might be delayed in those animals given 0.02 mg. For example, all birds injected with 20 mg synthesized 7S antibody by 7 days whereas those given 0.02 mg did not produce detectable 7S antibody until 14 days. Synthesis of 17S antibody also might be slightly delayed. By 14 days, all chickens possessed both 7S and 17S antibodies but at 35 days only 7S antibodies were found. Three of the 18 birds had 17S binding in the preimmune sera. Injection with FCA resulted in weakened conditions in some birds so that an inordinate number died (see section V). In fact, within 56 days after immunization, 5 of 18 birds died and by 490 days only 8 of 18 remained. These chickens still possessed 7S antibody although antigen binding appeared to be less intense in chickens 1738 and 1741. The surviving birds were given intravenous injections of 2 mg DNP-BGG 493 days after the primary injection

TABLE VIII

RELATIVE BINDING ACTIVITY[a] OF 17S AND 7S ANTI-BGG ANTIBODIES FROM CHICKENS IMMUNIZED WITH DNP-BGG IN FREUND'S COMPLETE ADJUVANT

Days following immunization	Chicken number									
	1250	1251	1252	1253	1254	1250	1251	1252	1253	1254
	17S antibody					7S antibody				
0[b]	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	+	0	0	0	0
8	+	+	0	0	+	++	++	+	0	+
11	0	+	0	0	+	++	++	+	+	+
18	0	0	0	0	0	++	++	0		+
22	0	0	0	0	0	++	++	0		+
34	0	0	0	0	0	++	++	0		+
2[c]	0	0	0	0	0	+	++	0	0	+
3	0	0	0	0	0	+	++	+	0	+
4	0	0	0	+		+	++	+	0	
6	0	0	0	+	0	+	++	+	0	+
8	0	0	0	+	0	+	++	+	0	+
11		0	0	+			++	++	++	
18	0	0	0	0	0	+	++	++	++	++
26	0	0	0	(-)[d]	0	+	++	++	(-)	++
35	0	0	0		0	+	++	++		++
56	0	0	0		0	+	++	++		++

[a] Relative binding activity as detected by radioimmunoelectrophoresis: 0 = no binding; + = weak binding; ++ = strong binding; blanks = not done.

[b] First injection: chickens immunized with 8 mg of DNP-BGG in FCA.

[c] Second injection: chickens boosted with 4 mg of DNP-BGG in FCA 34 days after the primary immunization.

[d] Chicken died.

Figure 6. The primary and secondary anti-BGG hemagglutination response of chickens given 2 intramuscular injections of DNP-BGG in Freund's complete adjuvant. The doses of the first and second injections were 8 and 4 mg of antigen, respectively. The interval between injections was 34 days.

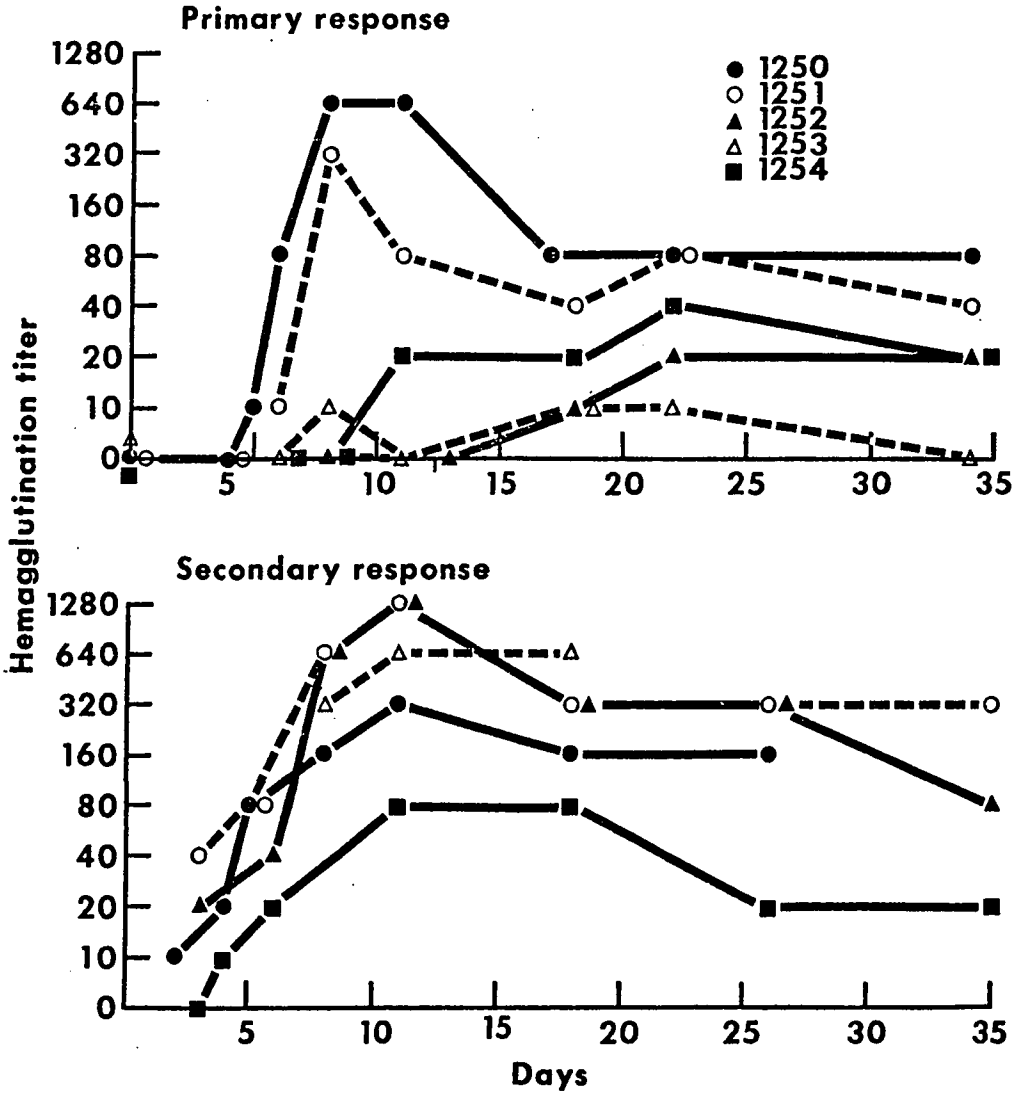

Primary response
1280
640
320
160
80
40
20
10
0
5
10
15
20
25
30
35
1250
1251
1252
1253
1254
Hemagglutination titer
Secondary response
1280
640
320
160
80
40
20
10
0
5
10
15
20
25
30
35
Days

TABLE IX

RELATIVE BINDING ACTIVITY OF 17S AND 7S ANTI-DNP ANTIBODIES FROM CHICKENS GIVEN VARIOUS DOSES OF DNP-BGG IN FREUND'S COMPLETE ADJUVANT

Chicken No.	Antigen (mg)	Days following immunization															
		0		3		7		9		14		35		490		499[b]	
		17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S
1734	0.02	0	0	0	0	0	0	0	0	+	+	0	++	(-)[c]			
1736		0	0	0	0	0	0	+	0	+	+	0	++	0	++	0	++
1737		0	0	0	0	0	0	+	0	+	+	0	++	(-)			
1738		0	0	0	0	0	0	+	0	++	++	0	++	0	+	0	++
1740	0.2	0	0	0	0	0	+	+	+	+	++	0	++	(-)			
1741		0	0	0	+	0	+	+	+	+	++	0	++	0	+	0	++
1742		+	0	+	+	+	+	0	+	+	++	0	++	(-)			
1743		+	0	+	0	+	+	+	+	++	++	0	++	(-)			
1744		0	0	0	0	+	+	+	+	0	++	0	++	0	++	0	++
1745	2	0	0	0	+	0	+	++	+	+	++	0	++	0	++	0	++
1746		0	0	0	0	+	+	+	+	++	++	0	++	0	++	0	++
1747		+	0	+	0	+	+	++	+	++	++	0	++	0	++	0	++
1748		0	0	0	0	+	+	++	+	++	++	0	++	(-)			
1749		0	0	0	0	+	+	+	+	++	++	0	++	(-)			
1750	20	0	0	0	0	+	++	+	++	++	++	0	++	(-)			
1751		0	0	0	0	0	++	+	++	+	++	0	++	(-)			
1752		0	0	+	+	+	++	+	++	+	++	0	++	0	++	0	++
1753		0	0	0	0	+	++	+	++	+	++	0	++	(-)			

[a] Relative binding activity as detected by radioimmunoelectrophoresis: 0 = no binding; + = weak binding; ++ = strong binding; blanks = not done.

[b] Surviving chickens were given an intravenous injection of 2 mg of DNP-BGG 493 days after priming.

[c] Chicken died.

to determine if 17S antibodies would form. Six days after boosting only intense 7S binding was found; no 17S antibody was evident (Table IX).

3. Intramuscular immunization with DNP-BGG emulsified in FIA

To determine if immune responses of chickens injected with FIA are qualitatively more like responses of birds immunized with FCA or more like those of birds given antigen in saline intravenously, groups of chickens were immunized with either 0.2 mg or 2.0 mg of DNP-BGG in FIA. Both 17S and 7S antibodies were present on the 9th and 14th days after immunization (Table X). By 21 days, only 7S antibodies were synthesized and these were detected for at least 4 months following immunization. Thus, the immune response of birds given FIA was qualitatively more like that of birds injected with FCA.

4. Repeated intravenous injections of antigen

The fact that one injection of antigen in FIA resulted in the continued synthesis of 7S antibodies raised the possibility that repeated intravenous injections of antigen might simulate this treatment. Therefore, three chickens were injected 14 times, every 4 days with 0.02 mg DNP-BGG in saline intravenously, and were bled immediately before each injection. Figure 7 depicts the RIE patterns obtained from these samples. The responses were variable. Both 7S and 17S antibodies of chicken serum 2157 had strong antigen binding activity from the 8th to the 32nd day and somewhat weaker 17S binding from 36 to 52 days after the first injection. Chicken serum 2158 showed strong binding by both antibodies throughout the series of injections starting from the 8th day.

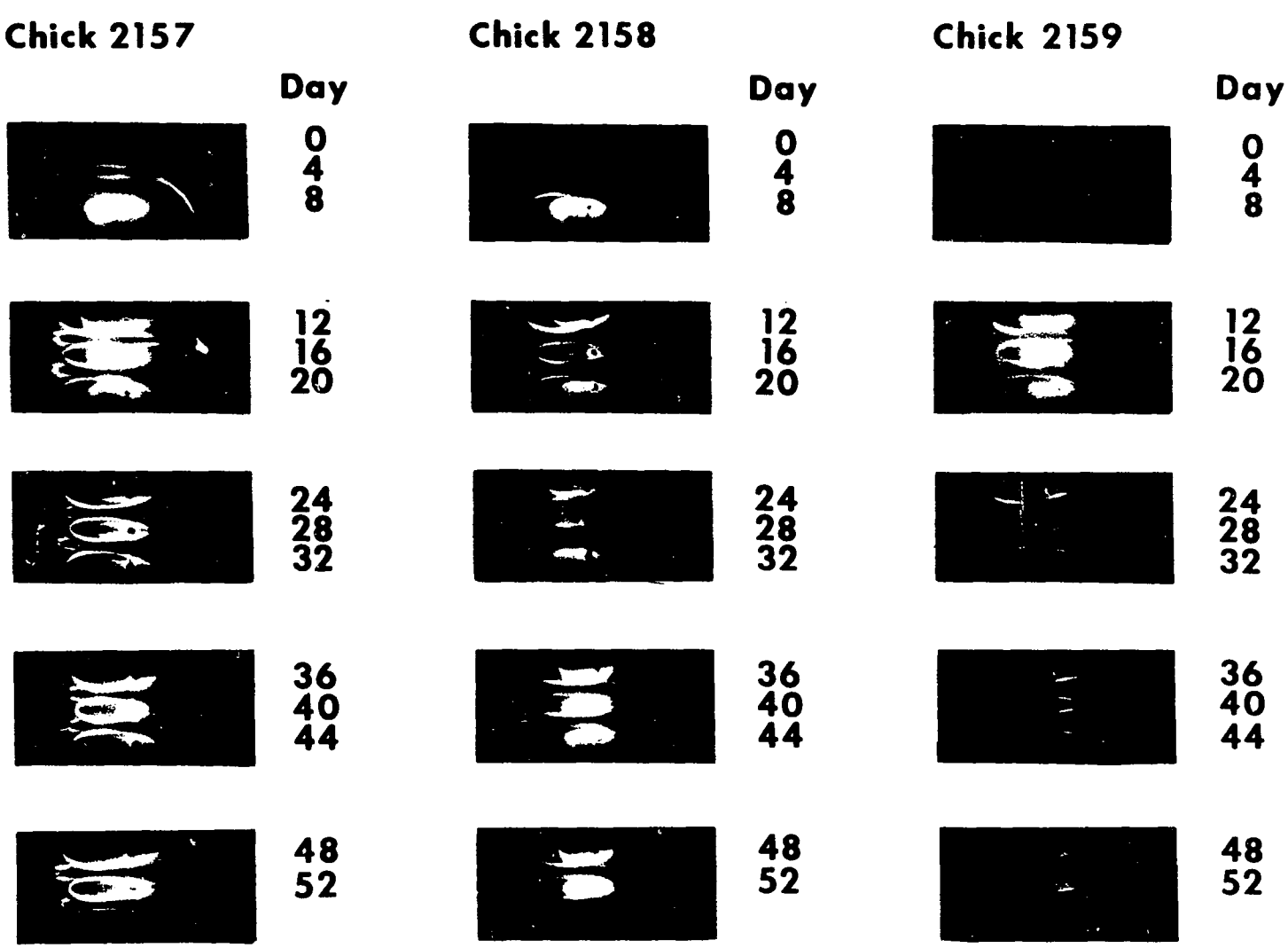
TABLE X

RELATIVE BINDING ACTIVITY[a] OF 17S AND 7S ANTI-DNP ANTIBODIES FROM CHICKENS IMMUNIZED WITH DNP-BGG IN FREUND'S INCOMPLETE ADJUVANT

Chicken No.	Antigen (mg)	Days following immunization											
		0		7		9		14		21		164	
		17S	7S	17S	7S	17S	7S	17S	7S	17S	7S	17S	7S
2025	0.2	0	0	0	0	+	0	+	+	0	++	0	++
2026		0	0	0	0	+	+	+	++	0	++	0	++
2027		0	0	0	0	+	+	+	+	0	++	0	++
2028	2	0	0	+	0	+	+	+	++	0	++	0	++
2029		+	0	+	0	+	+	+	++	0	++	0	++
2030		0	0	0	0	+	+	+	++	0	++	0	++

[a] Relative binding activity as detected by radioimmunoelectrophoresis: 0 = no binding; + = weak binding; ++ = strong binding.

Figure 7. The radioimmunoelectrophoretic patterns of sera obtained from chickens given 14 intravenous injections of 0.02 mg DNP-BGG each, every 4 days. Bleedings were taken just before injecting.


Chick 2157
Day
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Chick 2158
Day
0
4
8
12
16
20
24
28
32
36
40
44
48
52
Chick 2159
Day
0
4
8
12
16
20
24
28
32
36
40
44
48
52

Strong 7S and 17S binding was apparent for chicken 2159 on days 12 to 20. However, binding by both antibodies in this chicken was weaker on days 24 to 52. Despite the differences in these 3 birds, repeated antigen administration by the intravenous route did not stimulate the exclusive production of 7S antibody as had been observed in the adjuvant-treated birds.

B. Specific purification of anti-DNP antibodies

The binding properties of anti-hapten antibodies often have been determined using specifically purified antibodies. Also, in order to determine the valence, specifically purified antibody is required because the molar concentration of antibodies must be known. The feasibility of using specifically purified samples for following the affinity of antibodies from chickens given multiple intravenous injections of antigen and to determine the valence of the 7S and 17S antibodies was tested. Eight chickens were given intravenous injections of 2 mg of DNP-BGG each on 0, 52, 185 and 289 days. Serum pools were made from bleedings obtained 6, 7 and 8 days after each immunization. These will be referred to as 1°, 2°, 3° and 4° samples, respectively. The sera were adsorbed onto a DNP-lysyl Sepharose column and sequentially eluted first with DNP-OH, followed by DNP-glycine and finally with DNP-ε-aminocaproate. The fraction eluted with DNP-ε-aminocaproate was not studied since it contained considerable amounts of hapten which had remained firmly attached to the antibody binding sites. Also, insufficient antibody was obtained after one injection. The other specifically purified samples were fractionated into 7S and 17S antibodies by gel

filtration. Equilibrium dialysis was performed using hapten concentrations near saturation and Scatchard plots were constructed. Also included was a specifically purified 7S antibody preparation obtained from chickens which had received 2 intramuscular injections of DNP-BGG in FCA one month apart. In agreement with the results obtained by others (Gallagher & Voss 1969; Voss & Eisen 1972) who studied the 7S antibodies from a similar preparation, the valence extrapolated close to 2 (1.6) and had an affinity of about 1×10^6 M (Figure 8). The data obtained for antibodies from birds which had been given only intravenous injections showed marked heterogeneity as noted by the shape of the curves (Figure 8). Precise extrapolation to the x-axis was difficult to do. For DNP-OH eluted antibodies the valences were less than 0.8 and for DNP-glycine eluted antibodies the valences were less than 1.2. An accurate extrapolation is needed to determine the affinity constants. When the affinity constants from these curves were plotted as a function of different valences, an exponential curve was obtained (Figure 9). Thus, if the valence was assumed to be 2 the affinities obtained would be much lower than if an estimate was made by extrapolating to the x-axis. For example, for the DNP-glycine eluted, 3° antibody using a valence of 2.0, the $K_o = 1.1 \times 10^5 M^{-1}$ whereas using valence of 1.2, the K_o is 5 times higher ($5.2 \times 10^5 M^{-1}$). The fact that different valences were obtained for these preparations could be explained as follows: (1) residual hapten remained associated with the antibody especially those of high affinity; (2) considerable amounts of non-specific proteins or denatured protein were present; (3) relatively high concentrations of low affinity antibody ($k_o < 1 \times 10^4 M^{-1}$) was present in the population

Figure 8. Scatchard plots of specifically purified anti-DNP 7S antibody. The antibodies from serum pools were specifically purified by sequential elutions from an immunoadsorbent column with dinitrophenol, followed by DNP-glycine. The dose of all intravenous injections was 2 mg of DNP-BGG. Open circles = dinitrophenol-eluted antibody from sera obtained from chickens given 2 intravenous injections; open triangles = dinitrophenol-eluted antibody from sera obtained from chickens given 3 intravenous injections; open squares = dinitrophenol-eluted antibody from chickens given 4 intravenous injections: closed triangles = DNP-glycine-eluted antibody from chickens given 3 intravenous injections; closed squares = DNP-glycine-eluted antibody from chickens given 4 intravenous injections; closed circles = DNP-glycine-eluted antibody from chickens given 2 intramuscular injections each consisting of 8 mg of DNP-BGG in Freund's complete adjuvant.

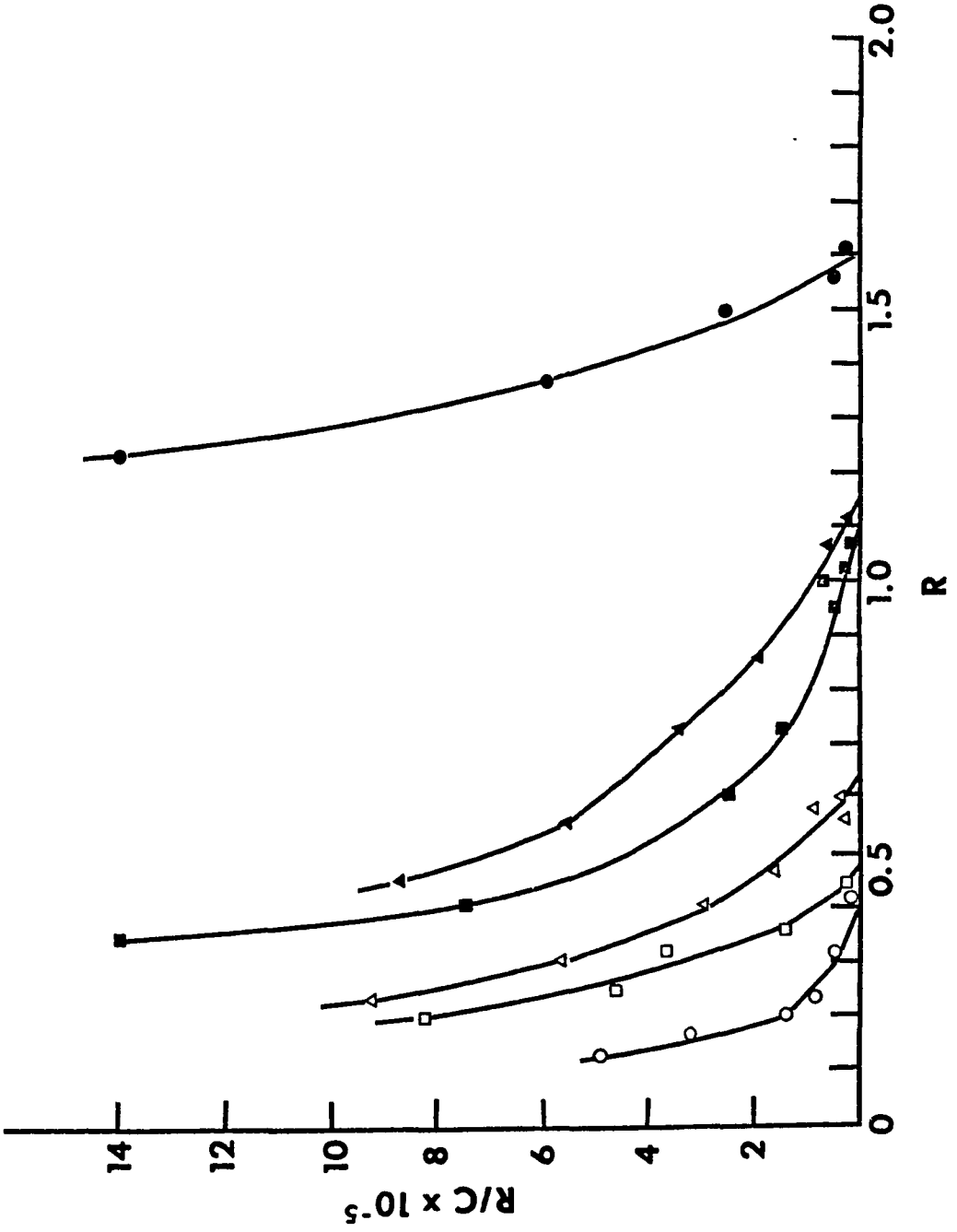
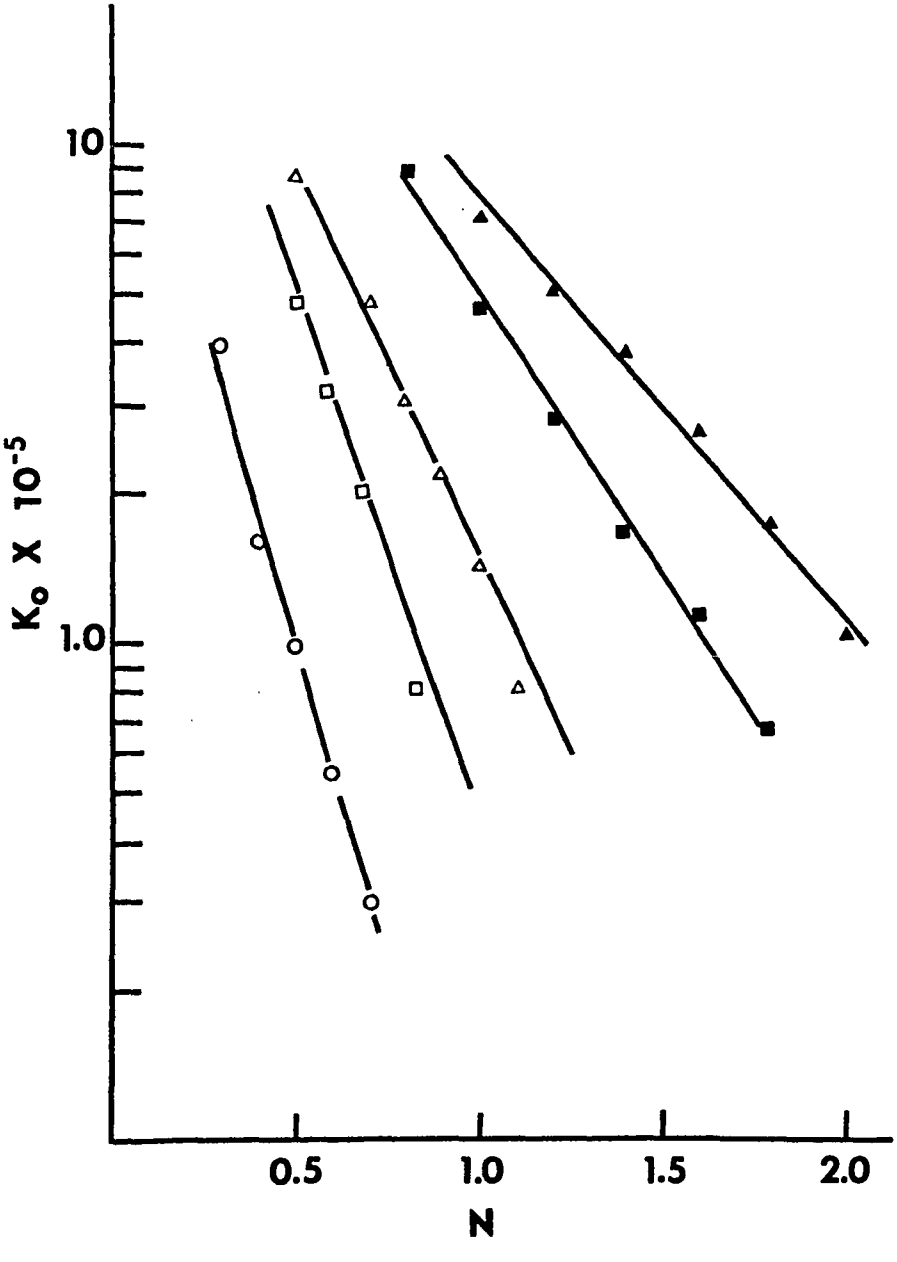

R
2.0
1.5
1.0
0.5
0
R/C x 10⁻⁵
14
12
10
8
6
4
2

Figure 9. A plot of the affinity constants versus the valence (N) from binding curves given in Figure 8. For different arbitrary values of N (N = R when $C \rightarrow \infty$), the point on the y-axis, R/C, which corresponds to N/2 was obtained. The affinity constant. $K_o = 1/C$ was calculated by multiplying (R/C)(1/R) where 1/R = 2/N. Open circles = dinitrophenol-eluted antibody from sera obtained from chickens given 2 intravenous injections; open triangles = dinitrophenol-eluted antibody from sera obtained from chickens given 3 intravenous injections; open squares = dinitrophenol-eluted antibody from chickens given 4 intravenous injections; closed triangles = DNP-glycine-eluted antibody from chickens given 3 intravenous injections; closed squares = DNP-glycine-eluted antibody from chickens given 4 intravenous injections. The dose of all intravenous injections was 2 mg of DNP-BGG.


10
1.0
K_o X 10^{-5}
0.5
1.0
1.5
2.0
N

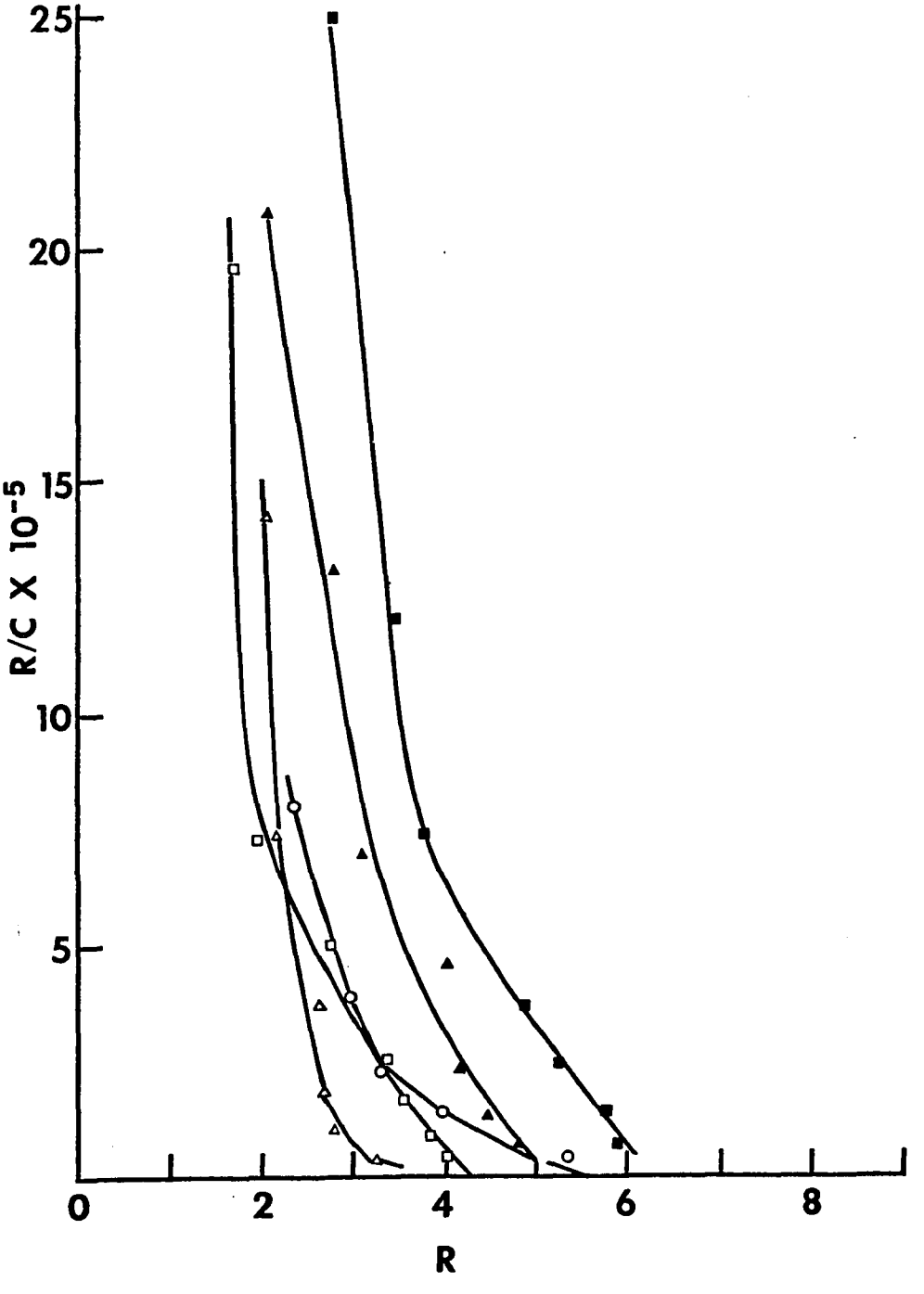
which could not be detected by equilibrium dialysis. In addition, specific purification might have selected low affinity antibody since high affinity antibody might not have been eluted with the various haptens and remained on the immunoadsorbent. Slightly higher valences and K_o values were obtained for the antibodies eluted with DNP-glycine. This might be due either to an actual selection of high affinity antibody or that less non-specific or denatured proteins were present. For these reasons it was decided that specific purification was not an acceptable method for determining antibody affinity in this study. The Scatchard plots of the 17S antibody are shown in Figure 10. A similar variation in antibody valence was noted. As will be discussed later (section V), it appears that values less than 10 could be accounted for by the same parameters that account for values of less than 2 for 7S antibody (see above).

C. Changes in antibody affinity during the immune response

1. Affinity of 7S antibody from chickens immunized with DNP-BGG in FCA

Chickens were given a single injection of either 0.02, 0.2, 2.0 or 20 mg of DNP-BGG emulsified in FCA and bled 7 to 9 days (1 week), 21 to 23 days (3 weeks), and 116 to 129 days (17 weeks) later. The 7S antibodies isolated by salt precipitation and Sephadex fractionation from the sera of 2 animals in each group were subjected to equilibrium dialysis studies using ^{3}H-DNP-lysine. Except for those chickens injected with 20 mg of antigen the concentrations of antibodies were insufficient from the other birds 1 week after immunization to perform

Figure 10. Scatchard plots of specifically purified anti-DNP 17S antibody obtained from the same serum pools as Figure 8. The antibodies were specifically purified by sequential elutions from an immunoadsorbent column with dinitrophenol, followed by DNP-glycine. Open circles = dinitrophenol-eluted antibody from sera obtained from chickens given 3 intravenous injections; open squares = dinitrophenol-eluted antibody from chickens given 4 intravenous injections; closed triangles = DNP-glycine-eluted antibody from chickens given 3 intravenous injections; closed squares = DNP-glycine-eluted antibody from chickens given 4 intravenous injections. The dose of all intravenous injections was 2 mg of DNP-BGG.


25
20
15
10
5
R/C X 10⁻⁵
0
2
4
6
8
R

binding studies. Early after immunization (3 weeks) the Sips plots were approximately linear for all preparations tested regardless of the dose of antigen used for injection (Figure 11). This indicates that, at this time, the antibody affinities were distributed in a symmetrical manner. However, at 17 weeks, the binding data of antibody preparations from chickens given low doses of antigen (0.02 or 0.2 mg) showed marked deviations from linearity in Sips plots. By comparing with previously published graphs and the histograms generated from them (Figure 1), these non-linear plots signify a skewing of the population towards high affinity antibodies. There was less apparent asymmetry of K_o values at 17 weeks of antibody obtained from birds injected with 2 and 20 mg of antigen. Thus the shift in the antibody population was dose dependent; low doses elicited a marked shift whereas higher doses did not.

Table XI lists $-\Delta F_1^\circ$ and $-\Delta F_2^\circ$ values calculated from the Sips plots. The 7S antibodies from chickens 1736 immunized with 0.02 mg increased in affinity having $-\Delta F_1^\circ$ values of 9.07 kcal/mole at 3 weeks and 12.29 kcal/mole at 17 weeks. This represents a 300-fold increase in K_o. The antibodies from chicken 1738 injected in the same manner showed a more moderate increase in $-\Delta F_1^\circ$ (9.30 to 10.82 kcal/mole). It is possible that the peak affinity had been reached before 17 weeks in this animal. The affinity of antibodies from chickens given 20 mg (chickens 1752 and 1753) at 17 weeks was lower when compared with the affinity of antibodies from birds injected with the lower doses although increases in K_o were apparent. The $-\Delta F_1^\circ$ values of chicken 1752 at 1, 3 and 17 weeks were 8.27, 7.70 and 8.40 kcal/mole, respectively; for chicken 1753, the values were 8.94, 7.71 and 9.55 kcal/mole, respectively. It is

Figure 11. Sips plots of binding data of anti-DNP 7S antibody from individual chickens (chicken number in upper left corners) given a single injection of DNP-BGG in Freund's complete adjuvant. The ordinate and abscissa are log $B/(B_t - B)$ and log C, respectively.

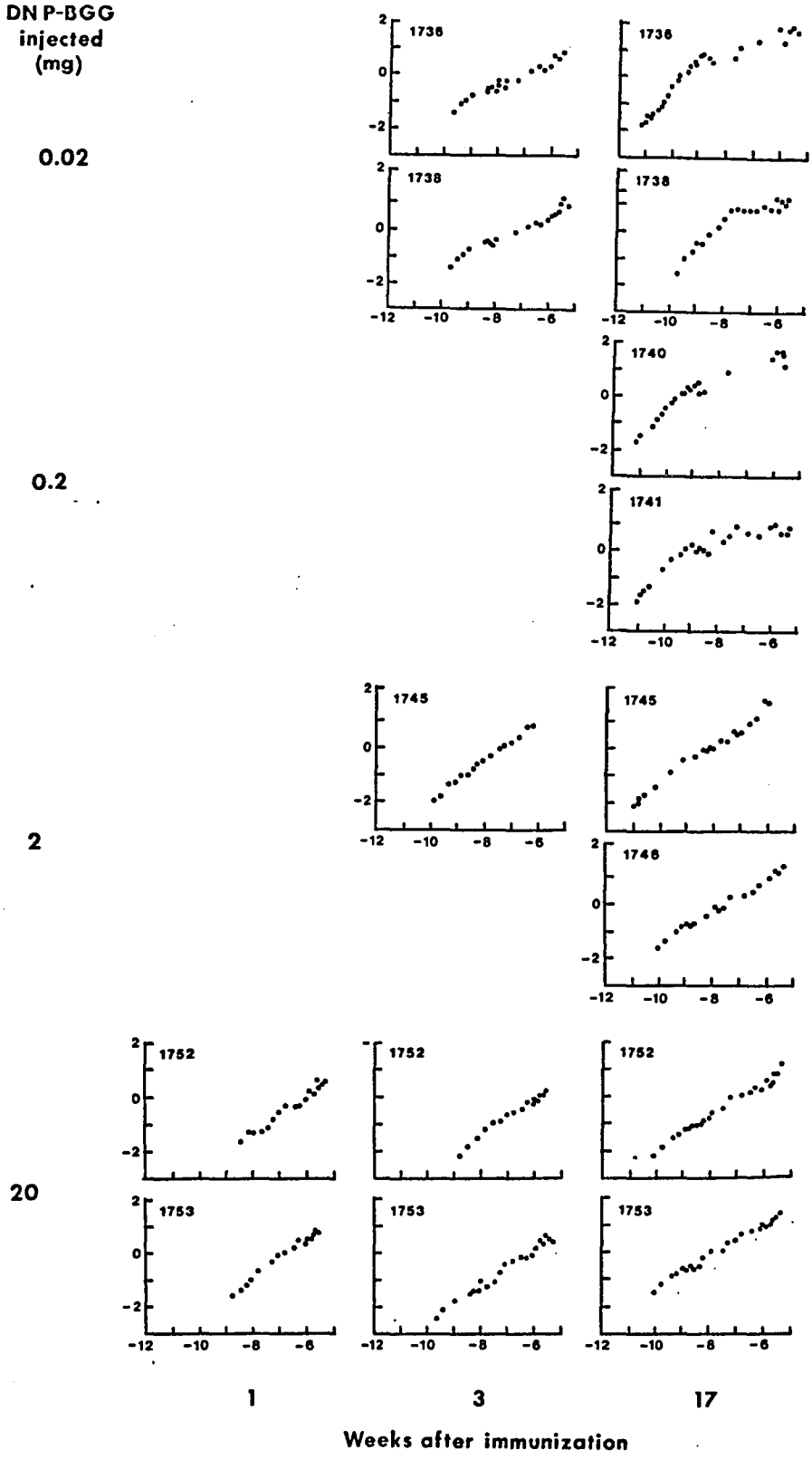

DN P-BGG injected (mg)
0.02
0.2
2
20
1736
1738
1740
1741
1745
1746
1752
1753
1
3
17
Weeks after immunization

TABLE XI

BINDING PROPERTIES[a] OF 7S ANTI-DNP ANTIBODY FROM CHICKENS IMMUNIZED WITH DNP-BGG IN FREUND'S COMPLETE ADJUVANT

Chicken No.	Weeks following immunization	Antigen (mg)	$-\Delta F_1^\circ$ (kcal/mole)	a_1	$-\Delta F_2^\circ$ (kcal/mole)	a_2
1736	3	0.02	9.07	.47	8.98	.45
	17		12.29	1.24	11.47	.60
1738	3	0.02	9.30	.51	9.11	.46
	17		10.82	1.18	9.82	.34
1740	3	0.2	(-)[b]			
	17		12.28	1.12	11.53	.50
1741	3	0.2	(-)			
	17		12.23	1.22	9.68	.40
1745	3	2	9.62	.84	8.07	.75
	17		11.23	.95	10.50	.69
1746	3	2	(-)			
	17		9.75	.63	9.66	.60
1752	1	20	8.27	.80	8.65	.73
	3		7.70	.77	7.46	.68
	17		8.40	.65	8.62	.61
1753	1	20	8.94	.94	8.56	.75
	3		7.71	.66	7.76	.67
	17		9.55	.50	9.80	.59

[a] $-\Delta F_1^\circ$ is the negative free energy change calculated from the Sips plot using the third of the total binding sites with the highest affinity; "a_1" is the heterogeneity index calculated from the slope of the Sips plot using a third of the sites; $-\Delta F_2^\circ$ and "a_2" are calculated using all of the sites.

[b] Not done.

interesting to note that there was a drop in affinity when the antibody obtained at 1 week was compared with that isolated 3 weeks after immunization in both birds. The non-linearity of the Sips plots observed in Figure 11 also is evident by comparing $-\Delta F_1^\circ$ values with $-\Delta F_2^\circ$ figures. The largest difference was observed in chicken 1741 (2.55 kcal/mole).

Although a detailed analysis throughout the immune response was not attempted and only a limited number of animals was used, maturation of affinity occurred using these conditions. No definitive statement on the rate of maturation can be made from these studies but the fact that chickens immunized with higher doses did not elicit high affinity antibody at 17 weeks indicates that the maturation process might be slower in these animals.

Analyses of the heterogeneity indices (Table XI) show that by using only a third of the total binding sites to calculate "a_1" there was restricted heterogeneity of antibody of high affinity. For example, at 17 weeks the antibody of chickens 1736, 1738, 1740 and 1745 had "a_1" values of 1.24, 1.18, 1.12 and .95, respectively. However, when all of the sites were included in the analysis heterogeneity was found. The above antibodies had "a_2" values of 0.60, 0.34, 0.50, 0.40 and 0.69, respectively. These results are best explained by assuming that a very restricted population of high affinity antibodies comprised the upper third of the total binding sites, whereas a wide range of affinities were found when all of the sites were considered. Values greater than 1 might be due, in part, to errors in the determination of the total concentration of binding sites, i.e., an overestimate of B_t resulted in an overestimate of "a" and an underestimate of K_o.

2. The affinity of 7S antibody from chickens immunized intravenously with DNP-BGG.

Since intravenous injections of DNP-BGG in saline yielded both 7S and 17S antibodies even after multiple injections (Table IV), studies were initiated to determine the K_o values of both classes of antibodies during the immune response. Chickens which had been immunized with a single intravenous injection of 2 mg of DNP-BGG did not produce enough antibody for affinity measurements by equilibrium dialysis when tested at a globulin concentration of 40 mg/ml. At least 2 injections at this dose were required. Because the booster response reflects the population of cells present immediately before the secondary stimulation, the protocol given in Table XII was used. Groups of birds were injected intravenously with various priming doses of antigen ranging from 0.02 to 20 mg. Chickens in each group were given second intravenous injections of 2 mg of DNP-BGG 1, 3, and 8 weeks after the first injection and bled 6, 7 and 8 days after the second injection. The globulins were precipitated and the 7S antibody from each bird was separated from the 17S antibody by two cycles of gel filtration on Sephadex G-200. It was anticipated that at some of the doses, the 7S antibody from chickens boosted 3 or 8 weeks after the primary injections would have a higher affinity than those boosted 1 week after priming. Contrary to expectation, binding data on the 7S antibodies obtained from all chickens, regardless of the dose or interval between injections, yielded approximately linear Sips plots (Figure 12). Obvious selection for high affinity antibody did not occur. The binding properties listed on Table XIII did not indicate consistent increases in antibody affinity for any

TABLE XII

IMMUNIZATION PROTOCOL FOR CHICKENS
GIVEN TWO INTRAVENOUS INJECTIONS OF DNP-BGG

Dose of first injection (mg)	Weeks between first and second injection[a]		
	1	3	8
0.02	1684[b]	1696	1708
	1685	1697	1709
	1686	1698	1710
0.2	1687	1699	1711
	1688	1700	1712
	1689	1701	1713
2	1690	1702	1714
	1691	1703	1715
	1692	1704	1716
20	1693	1705	1717
	1694	1706	1718
	1695	1707	1719

[a] Dose of second injection was 2 mg of DNP-BGG; all chickens were bled 6, 7 and 8 days after the second injection.

[b] Chicken number.

Figure 12. Sips plots of binding data of anti-DNP 7S antibody from individual chickens (chicken number in upper left corners) given 2 intravenous injections of DNP-BGG. The ordinate and abscissa are log $B/(B_t - B)$ and log C, respectively. Chickens were primed with the doses indicated. A second intravenous injection of 2 mg of antigen was given at either 1, 3 or 8 weeks later.

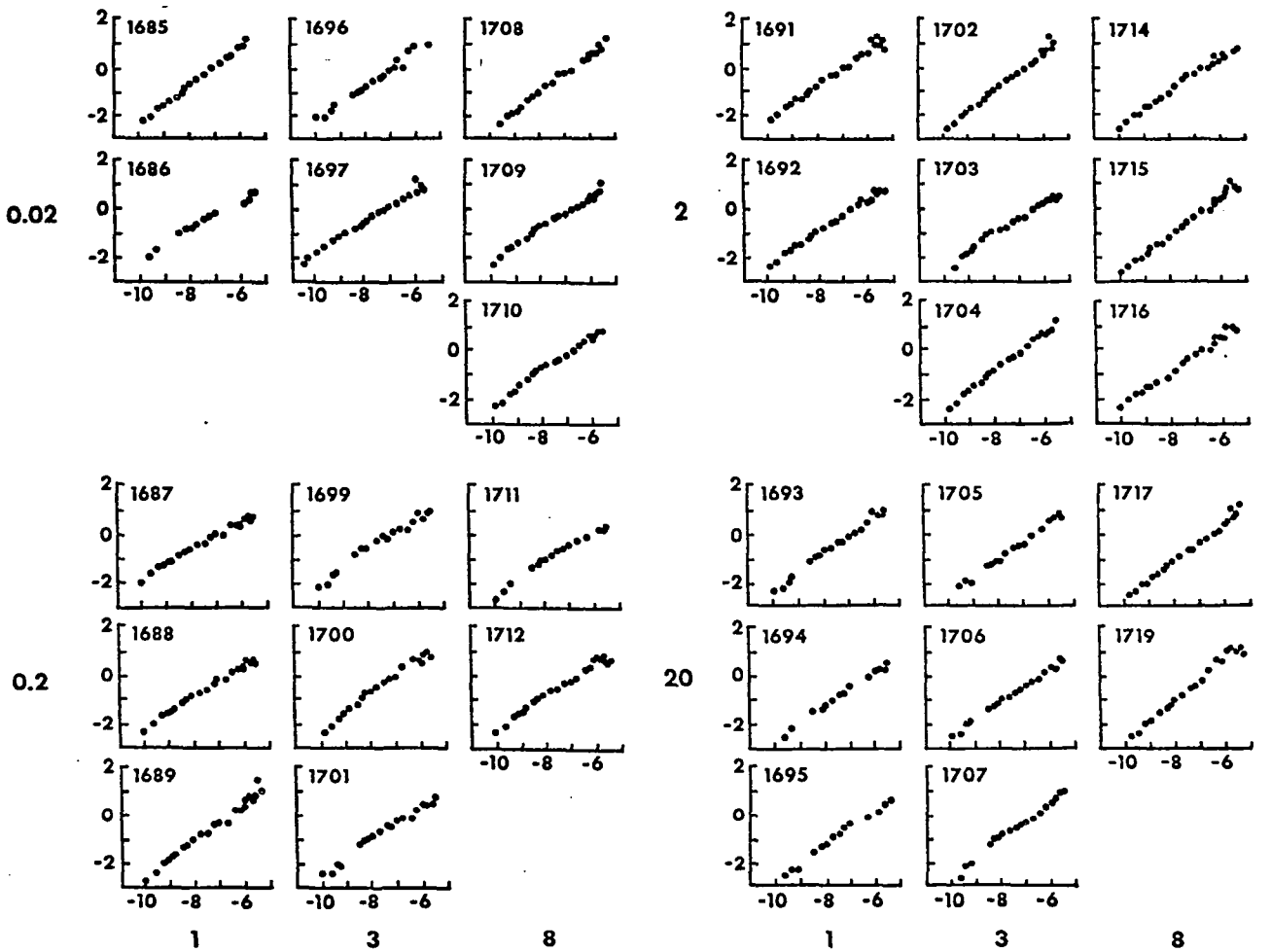

0.02
2
0.2
20
1685
1696
1708
1686
1697
1709
1710
1691
1702
1714
1692
1703
1715
1704
1716
1687
1699
1711
1688
1700
1712
1689
1701
1693
1705
1717
1694
1706
1719
1695
1707
2
0
-2
-10
-8
-6
1
3
8
Interval between injections (weeks)

TABLE XIII

BINDING PROPERTIES[a] OF 7S ANTI-DNP ANTIBODY FROM CHICKENS GIVEN TWO INTRAVENOUS INJECTIONS OF DNP-BGG

First injection of antigen (mg)	Weeks between injection[b]	Chicken No.	$-\Delta F^\circ_1$ (kcal/mole)	a_1	$-\Delta F^\circ_2$ (kcal/mole)	a_2
0.02	1	1684				
		1685	8.95	.82	8.94	.81
		1686	8.79	.70	8.47	.59
		$\bar{x}$[c]	8.87		8.70	
	3	1696	8.66	.58	8.72	.70
		1697	9.20	.67	9.17	.68
		1698	(-)[d]			
		$\bar{x}$	8.93		8.94	
	8	1708	8.66	.82	8.69	.84
		1709	9.04	.84	8.62	.69
		1710	8.97	.80	8.73	.72
		$\bar{x}$ ± S.D.[c]	8.89 ± .20		8.68 ± .05	
0.2	1	1687	8.92	.64	8.66	.54
		1688	8.59	.71	8.30	.64
		1689	8.47	.77	8.53	.82
		$\bar{x}$ ± S.D.	8.66 ± .23		8.50 ± .18	
	3	1699	9.48	.92	9.10	.80
		1700	9.25	.91	8.88	.76
		1701	8.85	.84	8.43	.72
		$\bar{x}$ ± S.D.	9.19 ± .32		8.80 ± .34	
	8	1711	8.47	.78	8.15	.66
		1712	8.98	.74	8.46	.62
		1713	(-)			
		$\bar{x}$	8.72		8.30	
2.0	1	1690				
		1691	8.94	.75	8.92	.73
		1692	8.61	.76	8.43	.73
		$\bar{x}$	8.78		8.68	
	3	1702	8.79	.84	8.66	.82
		1703	8.43	.74	8.22	.68
		1704	8.94	.85	8.79	.81
		$\bar{x}$ ± S.D.	8.72 ± .26		8.56 ± .30	
	8	1714	8.79	.81	8.41	.72
		1715	8.41	.80	8.47	.80
		1716	8.42	.70	8.60	.72
		$\bar{x}$ ± S.D.	8.52 ± .22		8.49 ± .10	
20	1	1693	8.60	.81	8.73	.76
		1694	7.90	.71	7.83	.69
		1695	8.34	.84	8.15	.78
		$\bar{x}$ ± S.D.	8.28 ± .35		8.24 ± .46	
	3	1705	8.47	.80	8.45	.79
		1706	8.27	.72	8.15	.70
		1707	8.81	.92	8.56	.81
		$\bar{x}$ ± S.D.	8.52 ± .27		8.39 ± .21	
	8	1717	8.30	.74	8.40	.76
		1718				
		1719	8.83	.85	8.98	.90
		$\bar{x}$	8.56		8.69	

[a] $-\Delta F^\circ_1$ is the negative free energy change calculated from the Sips plot using the third of the total binding sites with the highest affinity; "a_1" is the heterogeneity index calculated from the slope of the Sips plot using a third of the sites; $-\Delta F^\circ_2$ and "a_2" are calculated using all of the sites.

[b] Interval between primary and secondary injections; the dose of the second injection was 2 mg of DNP-BGG; antibody was isolated from serum obtained 6, 7 and 8 days after boosting.

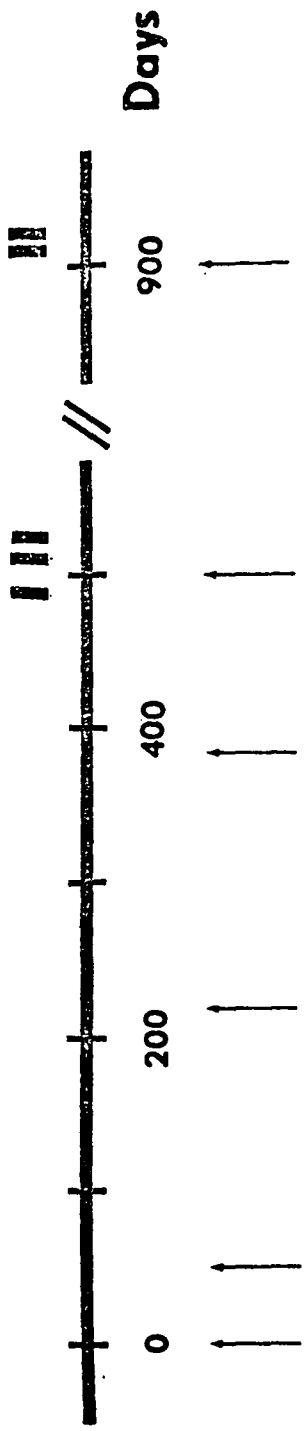
[c] Refers to mean and standard deviation.

[d] Insufficient 7S antibody present in the globulin preparation.

of the groups of chickens followed for 8 weeks. The differences in either mean $-\Delta F_1^\circ$ values or mean $-\Delta F_2^\circ$ values between any 2 groups were less than 1 kcal/mole and the maximum difference between any 2 individual chickens (no. 1699 versus no. 1694) was 1.58 kcal/mole. Although not enough animals within each of the groups were tested to allow for meaningful statistical analysis differences less than 1 kcal/mole most probably are not significant expecially since there are 3 sources of variability: (1) experimental error; (2) biological variations among animals; and (3) errors inherent in the technique. In regard to the last point, Werblin & Siskind (1972b) showed by using a hypothetical model of low affinity antibodies that B_t is underestimated resulting in an overestimate in $-\Delta F$ of 0.3 kcal/mole when the binding method used in this study is imployed. However, antibodies of higher affinities might be induced if other samples were tested. For example, perhaps the 8-week interval was too short for the full development of the maturation process. Higher K_o might be obtained if the interval between injections were lengthened. Antibody isolated late after immunization from an individual animal which had been given multiple intravenous injections might show increases in affinities when compared with antibody isolated earlier. Since different chickens were used at each time, increases may have been missed. Perhaps a dose lower than 2 mg should be used for the booster injection.

In an attempt to study these possibilities, the following was done. Three birds were given 6 intravenous injections. The doses of the first 5 injections were 2 mg each, and for the last injection the dose was 0.1 mg. As shown in Figure 13, antigen was administered on 0, 52, 218,

Figure 13. Immunization protocol and bleeding schedule for chickens given multiple intravenous injections. Arrows refer to the day of injection and bars refer to the days of bleeding.


Days
900
400
200
0

382, 501, and 901 days. The 7S antibody was isolated from serum obtained on the following days after the first injection: 489 to 495 days (16 week, 4°); 507 to 511 days (1 week, 5°); 522 to 525 days (3 week, 5°); 907 to 909 days (1 week, 6°) and 922 to 925 days (3 week, 6°). Some of the binding data are shown in Figure 14 in the form of Sips plots. Marked deviations from linearity were not observed although slight deviations were noted. Chicken 1544 did not have sufficient antibody late after the 4th injection or 3 weeks after the 5th injection for equilibrium dialysis studies. The data for samples obtained 1 and 3 weeks after the 6th injection are incomplete. The binding properties calculated from the plots are listed in Table XIV. The K_o of antibody obtained 1 week after 5 injections were slightly higher than that of antibody obtained before boosting. The $-\Delta F_1^\circ$ antibodies from chickens 1540 and 1541 increased from 8.02 to 8.72 kcal/mole and 8.15 to 8.81 kcal/mole, respectively. Three weeks after the fifth injection, chickens 1540 and 1541 produced antibodies which had $-\Delta F_1^\circ$ values of 8.11 and 7.12, kcal/mole, respectively. After a 6th injection antibodies of low K_o continue to be synthesized (data are not shown). Because of the limited number of chickens used, it cannot be established if the slight increases and decreases in affinities are significant. Nevertheless, maturation of antibody affinity, like that observed in chickens immunized with antigen in FCA did not occur. The $-\Delta F$ values of antibodies isolated from chickens 16 weeks after 4 injections, 3 weeks after 5 injections or after immunizing with 0.1 mg of antigen were not greater than 10 kcal/mole.

Figure 14. Sips plots of binding data of anti-DNP 7S antibody from chickens (chicken number in upper left corners) after multiple intravenous injections of DNP-BGG. Sera were obtained 16 weeks after 4 intravenous injections (A), 1 week after 5 injections (B) and 3 weeks after 5 injections (C). The dose for each injection was 2 mg.

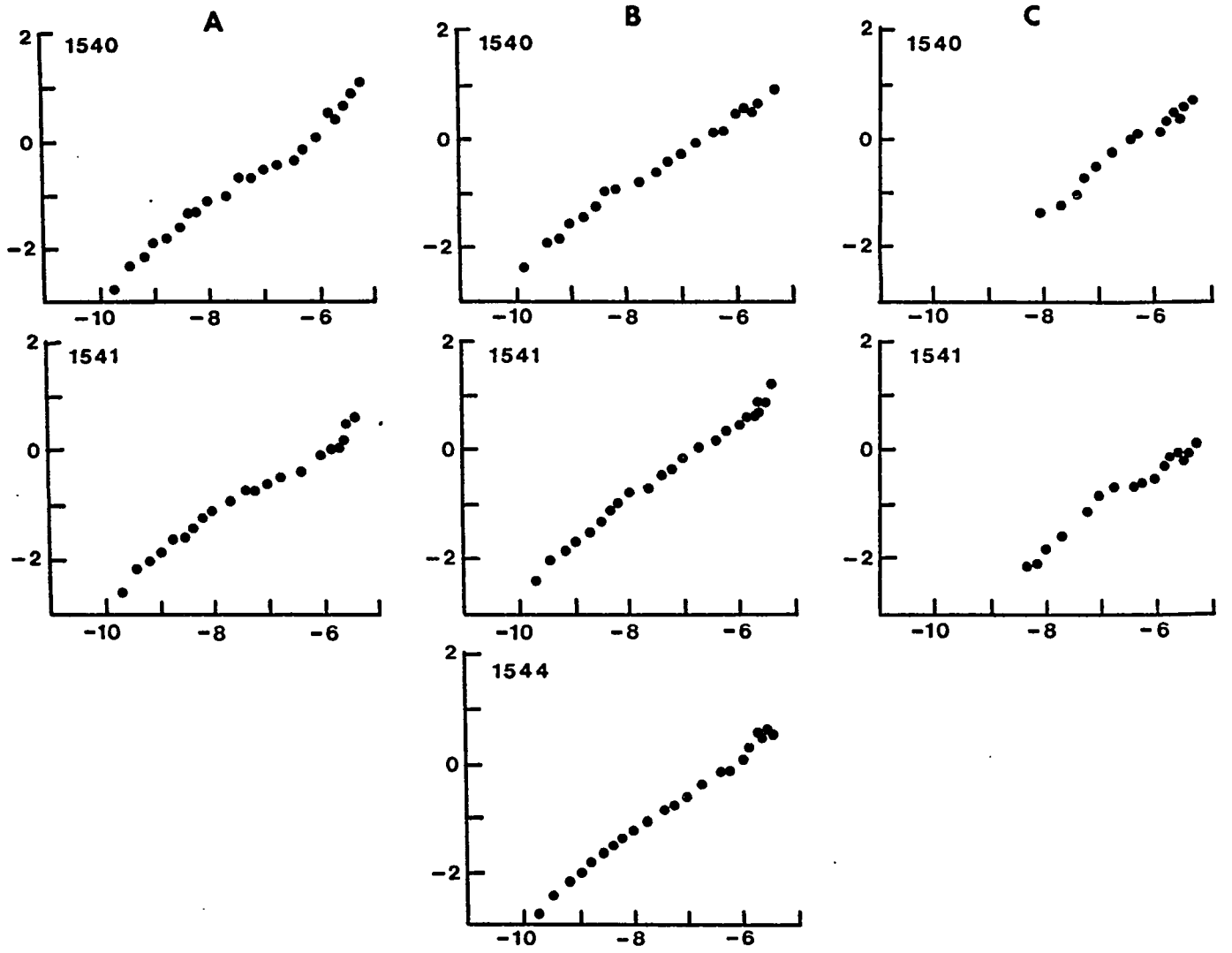

A
B
C
1540
1541
1544
2
0
-2
-10
-8
-6

TABLE XIV

BINDING PROPERTIES[a] OF 7S ANTI-DNP ANTIBODIES FROM CHICKENS GIVEN MULTIPLE INTRAVENOUS INJECTIONS OF DNP-BGG

Chicken No.	Number of injections[b]	Weeks after last injection	$-\Delta F_1^\circ$ (kcal/mole)	a_1	$-\Delta F_2^\circ$ (kcal/mole)	a_2
1540	4	16	8.02	0.72	7.98	0.73
	5	1	8.72	0.76	8.47	0.69
	5	3	8.11	0.84	7.98	0.77
1541	4	16	8.15	0.64	7.70	0.63
	5	1	8.81	0.83	8.59	0.76
	5	3	7.12	0.72	7.00	0.72
1544	4	16	(-)[c]			
	5	1	8.87	0.76	8.80	0.75
	5	3	(-)			

[a] $-\Delta F_1^\circ$ is the negative free energy change calculated from the Sips plot using the third of the total binding sites with the highest affinity; "a_1" is the heterogeneity index calculated from the slope of the Sips plot using a third of the sites; $-\Delta F_2^\circ$ and "a_2" are calculated using all of the sites.

[b] Intravenous injections with 2 mg of DNP-BGG.

[c] Insufficient 7S antibody present in the globulin preparation.

3. Affinity of 7S antibody from chickens immunized with antigen in FIA

Based on RIE (Table X), the immune responses of birds immunized with DNP-BGG in FIA resembled responses of animals given antigen in FCA rather than those of intravenously-injected birds. To determine whether or not the K_o values reflected this observation, birds were given a single injection of either 0.2 or 2.0 mg of antigen in FIA and bled 91 to 93 days (13 weeks) later. The Sips plot of the binding data from chicken 2026 was approximately linear (Figure 15). However, the plots of antibodies from the other 3 chickens (No. 2027, 2028 and 2029) showed considerable deviation from linearity indicating that a selection for high affinity antibody occurred. In all cases, the $-\Delta F_1^\circ$ values were high (Table XV) ranging from 10.44 to 10.88 kcal/mole. Some restriction in the high affinity antibody population of chickens 2028 and 2029 was evident from the heterogeneity index, "a_1," with values of 0.86 and 0.75, respectively.

4. Comparison of 7S and 17S antibody affinities

The immune responses of chickens which had been injected with DNP-BGG intravenously was characterized previously by the persistent production of both 7S and 17S antibodies. Therefore, the binding properties of these two antibodies obtained from individual animals were compared. The 7S and 17S antibodies from chickens given 2 intravenous injections according to the protocol in Table XII were studied. Figure 16 presents 12 binding curves for the 17S antibody and the curves for the 7S antibodies which were depicted earlier in Figure 12. Except for chickens

Figure 15. Sips plots of binding data of anti-DNP 7S antibody from chickens given a single intramuscular injection of DNP-BGG in Freund's incomplete adjuvant. The ordinate and abscissa are log $B/(B_t - B)$ and log C, respectively. Chickens 2026 and 2027 were given 0.2 mg of antigen and chickens 2028 and 2029 were given 2 mg of antigen. Antibody was isolated 13 weeks after immunization.

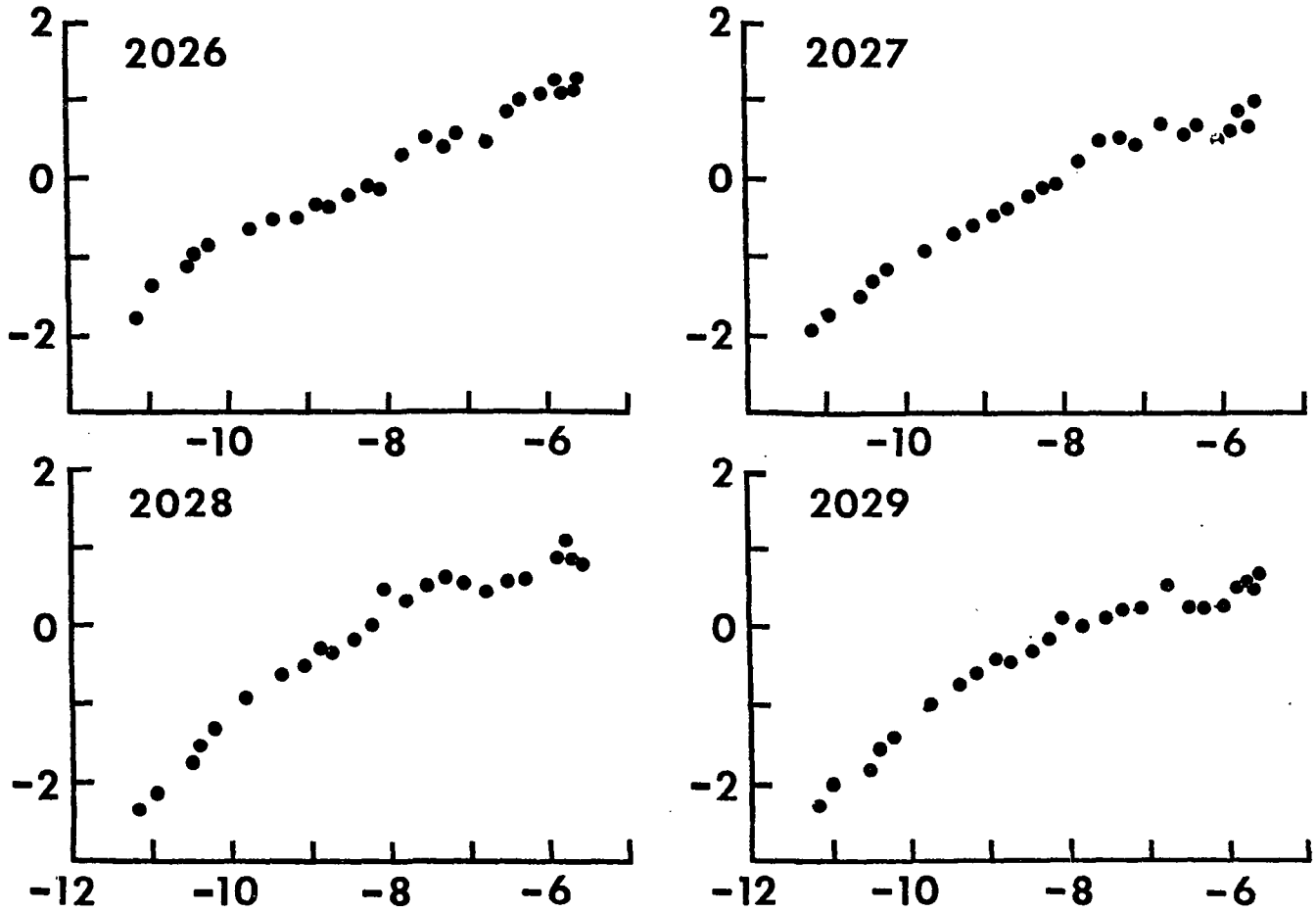

2026
2027
2028
2029
2
0
-2
-10
-8
-6
-12

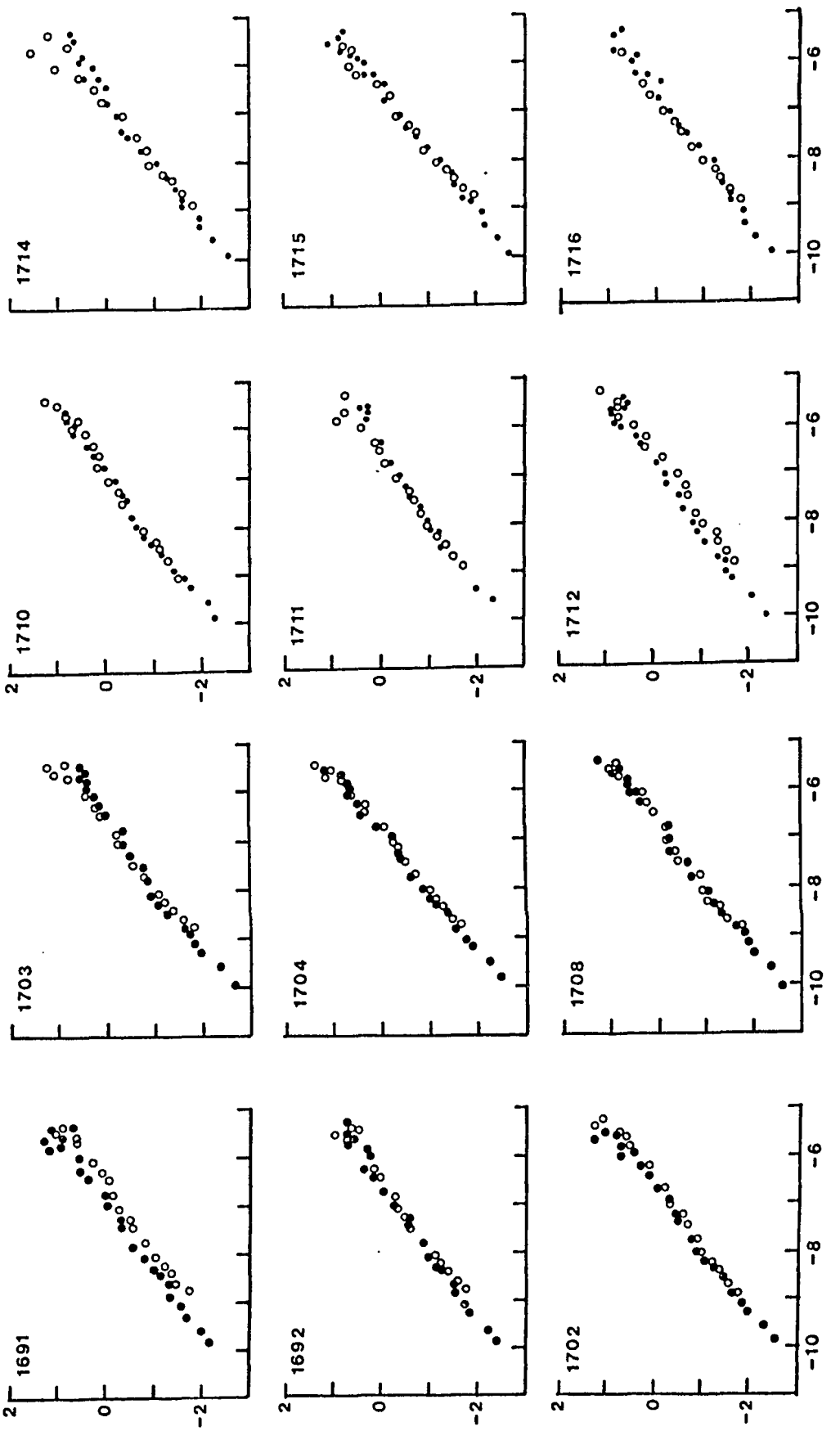
TABLE XV

BINDING PROPERTIES[a] OF 7S ANTI-DNP ANTIBODIES OBTAINED FROM CHICKENS 13 WEEKS AFTER INTRAMUSCULAR INJECTION OF DNP-BGG IN FREUND'S INCOMPLETE ADJUVANT

Chicken No.	Antigen (mg)	$-\Delta F_1^\circ$ (kcal/mole)	a_1	$-\Delta F_2^\circ$ (kcal/mole)	a_2
2026	0.2	10.63	0.52	10.40	0.50
2027	0.2	10.44	0.62	9.80	0.48
2028	2.0	10.88	0.86	10.00	0.55
2029	2.0	10.51	0.75	9.29	0.47

[a] $-\Delta F_1^\circ$ is the negative free energy change calculated from the Sips plot using the third of the total binding sites with the highest affinity; "a_1" is the heterogeneity index calculated from the slope of the Sips plot using a third of the sites; $-\Delta F_2^\circ$ and "a_2" are calculated using all of the sites.

Figure 16. Sips plots comparing the binding data of anti-DNP 7S (closed circles) and 17S (open circles) antibody from individual chickens (chicken number in upper left) given 2 intravenous injections of DNP-BGG. The ordinate and abscissa are log $B/(B_t - B)$ and log C, respectively. The 7S curves were given in Figure 12.


1691
1692
1702
1703
1704
1708
1710
1711
1712
1714
1715
1716
2
0
-2
-6
-8
-10

1691 and 1712, the 17S binding data can be almost superimposed on the 7S curves. The largest difference between $-\Delta F_1^\circ$ values of 7S and 17S antibody affinities was 0.83 kcal/mole for chicken 1712 (Table XV). The other differences were within 0.5 kcal/mole of each other. The heterogeneity indices of the 7S and 17S antibodies were similar although high values for 17S antibodies were obtained for chicken 1714 ("a_2" = 0.94) and chicken 1715 ("a_1" = 0.93). Thus, despite the different doses of antigen and the interval between injections no consistent differences were found in individual animals between the binding properties of 7S and 17S antibodies. Under these immunization protocols striking increases in antibody affinities were not found for either the 7S or 17S antibodies.

5. Concentrations of 7S and 17S antibodies after intravenous injections

The total concentration of antibody was determined from a plot of 1/B versus 1/C. The 7S antibody was expressed as the concentration of antibody per 10 mg of 7S globulin and the 17S antibody as the concentration per 1 mg of 17S globulin. The assumptions and advantages of this method will be described later. The amounts of 7S and 17S antibodies showed considerable variations (Figure 17) although the quantities were consistently low. Other than chicken 1696 which had an unusually low amount of antibody (0.01 mg/10 mg globulin), there was over a 10-fold difference (0.02 to 0.28/10 mg 7S globulin) between the amounts of antibody of the remaining 30 chickens. There appears to be more antibody produced when the interval between injections is short (1 week)

TABLE XVI

COMPARISON OF BINDING PROPERTIES[a] OF 7S AND 17S ANTIBODIES

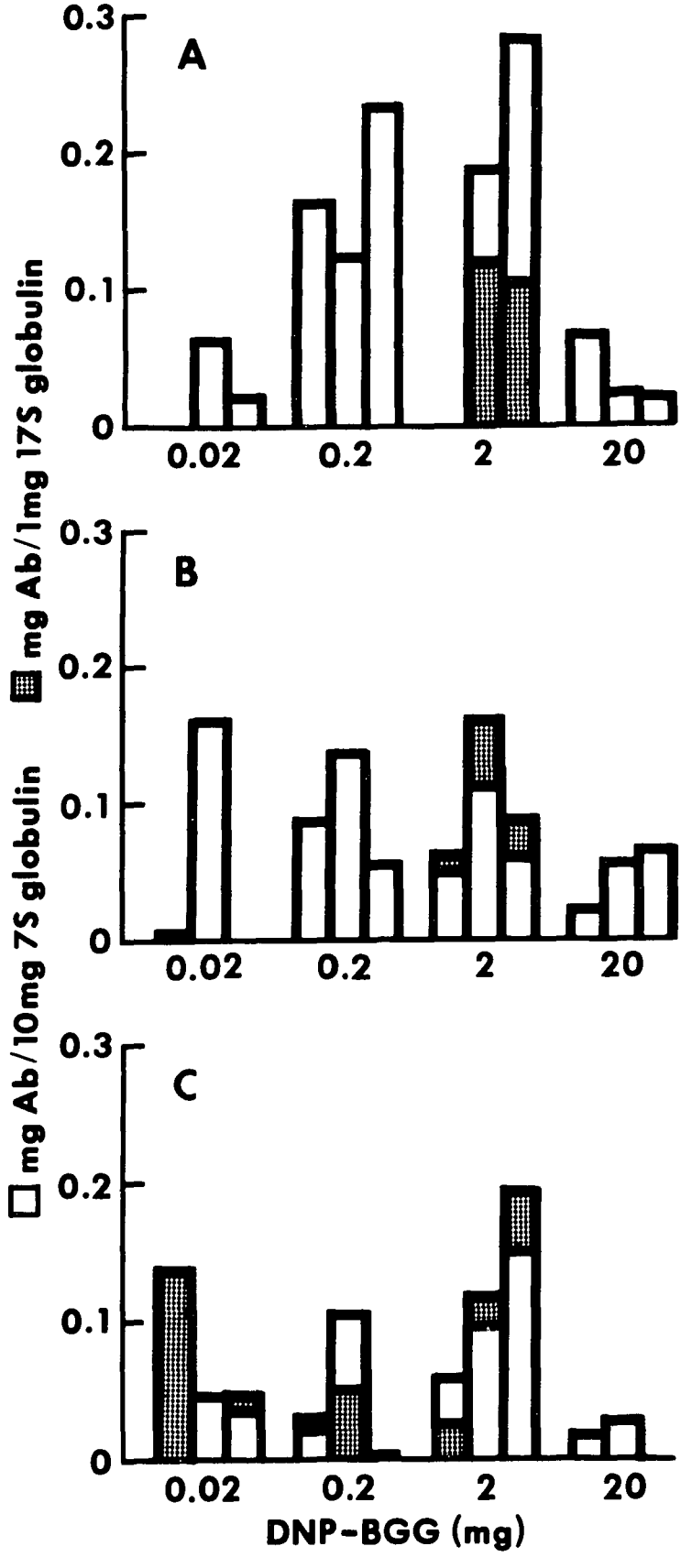
Chicken No.	Antigen[b] (mg)	Interval[c] between injections (weeks)	$-\Delta F_1^\circ$ (kcal/mole)		a_1		$-\Delta F_2^\circ$ (kcal/mole)		a_2	
			7S	17S	7S	17S	7S	17S	7S	17S
1691	2.0	1	8.94	8.63	0.75	0.84	8.92	8.30	0.73	0.77
1692	2.0	1	8.61	8.72	0.76	0.89	8.43	8.38	0.73	0.79
1702	2.0	3	8.79	8.34	0.84	0.71	8.66	8.40	0.82	0.78
1703	2.0	3	8.43	8.68	0.74	0.89	8.22	8.53	0.68	0.82
1704	2.0	3	8.94	8.81	0.85	0.87	8.79	8.85	0.81	0.88
1708	0.02	8	8.66	8.72	0.82	0.82	8.69	8.53	0.84	0.73
1710	0.02	8	8.98	8.78	0.80	0.77	8.73	8.65	0.72	0.70
1711	0.20	8	8.47	8.34	0.78	0.71	8.15	8.55	0.66	0.76
1712	0.20	8	8.98	8.15	0.74	0.64	8.46	8.50	0.62	0.78
1714	2.0	8	8.79	8.53	0.81	0.76	8.41	8.83	0.72	0.94
1715	2.0	8	8.41	8.27	0.80	0.93	8.47	8.52	0.80	0.88
1716	2.0	8	8.42	8.85	0.70	0.86	8.60	8.82	0.72	0.85

[a] $-\Delta F_1^\circ$ is the negative free energy change calculated from the Sips plot using the third of the total binding sites with the highest affinity; "a_1" is the heterogeneity index calculated from the slope of the Sips plot using a third of the sites; $-\Delta F_2^\circ$ and "a_2" are calculated using all of the sites.

[b] Dose of primary injection.

[c] Interval between primary and secondary injections; the dose of the second injection was 2 mg of DNP-BGG; antibody was isolated from serum obtained 6, 7 and 8 days after boosting.

Figure 17. Relative concentrations of 7S and 17S anti-DNP antibody from chickens given 2 intravenous injections of DNP-BGG. Chickens were primed with the doses indicated. A second intravenous injection of 2 mg of antigen was given either 1 week (A), 3 weeks (B) or 8 weeks (C) later. Each bar represents an individual chicken. Concentrations of antibody were determined from equilibrium measurements of either 10 mg of 7S or 1 mg of 17S globulin preparations. The total binding sites were determined by extrapolation to the y-axis from a 1/B versus 1/C plot. The reciprocal of that value was multiplied either by 1.7×10^5 (molecular weight of 7S Ig)/2 (valence of 7S antibody) for 7S antibody or by 8.9×10^5 (molecular weight of 17S Ig)/10 (valence of 17S antibody) for 17S antibody.


A
B
C
0.3
0.2
0.1
0
0.02
0.2
2
20
mg Ab/1mg 17S globulin
mg Ab/10mg 7S globulin
DNP-BGG (mg)

than when the interval is longer (3 to 8 weeks). For those birds primed with 0.2 or 2 mg of antigen and boosted at 1 week the range of antibody concentrations was 0.12 to 0.28/10 mg globulin. At an interval of 3 weeks between injections, the range was 0.05 to 0.14 and at an interval of 8 weeks, the range was 0.02 to 0.015 mg/10 mg globulin. Furthermore less antibody was produced with very low (0.02 mg) or very high (20 mg) doses of priming antigen than with intermediate immunizing doses when a 1-week interval between injections was used. Thus, the 2 birds immunized with 0.02 mg had concentrations of 7S antibodies of 0.06 and 0.02 mg/10 mg globulins and the 3 chickens injected with 20 mg had 0.08, 0.03 and 0.02 mg antibody/10 mg globulin. In contrast, the range of concentrations for chickens injected with 0.2 or 20 mg was high (0.12 to 0.28 mg/10 mg globulin). This relationship was not as apparent at injection intervals of 3 weeks or 8 weeks. The data for the 17S antibody are expressed as the amount of 17S antibody/1 mg 17S globulin. On a molar basis, less 17S antibody was produced when compared with the concentration of 17S antibody. The molar ratio of 7S to 17S antibody varied from 3 to 14.

Generally the amounts of 17S antibody paralleled the amounts of 7S antibody, i.e., when there were low amounts of 7S antibody there were also low amounts of 17S antibody (Figure 17).

V. DISCUSSION

The primary response of chickens given an intravenous injection of DNP-BGG was characterized by the transient synthesis of 7S and 17S antibodies. Both classes of antibody bound antigen by 6 days as revealed by RIE. The titers by PHA of the primary antisera were low and high-salt precipitins were not detected. One month after immunization little or no antibodies were found. These results were similar to those reported by Orlans et al (1968) who observed that chickens given a single intravenous injection of DNP-BGG did not produce hemagglutination or precipitating antibodies. However, we established that a second intravenous injection 34 days after the first resulted in the production of 7S and 17S antibodies both of which bound antigen intensely by RIE. After 2 injections, the PHA titers were high and all sera contained high-salt precipitins by the 4th day. The amounts of 7S and 17S antibodies were determined from 12 chickens which had been primed with various doses of DNP-BGG (0.02 to 2 mg) and boosted with 2 mg at 1, 3 or 8 weeks later. The concentration of 7S antibody found in the serum was greater than the concentration of 17S antibodies and the molar ratio of 7S:17S varied from 3 to 14. By RIE, weak 7S binding and intense 17S activity was apparent 134 days after the second injection. A third intravenous injection of DNP-BGG still resulted in the synthesis of both classes of antibody. In fact, when 14 injections were given every 4 days to 3 chickens, all had variable but detectable 7S and 17S antibodies which bound to antigen. The minimum dose for eliciting a primary response by the intravenous route of injection was about 0.05 mg of DNP-BGG. At this dose, 3 of 5 chickens did not

demonstrate binding antibody by RIE. However, it was sufficient to prime for a booster response since a second injection resulted in the production of both 7S and 17S antibody which was qualitatively indistinguishable from that obtained from birds given higher doses. This finding was similar to that of Valentova et al (1967) who found that chickens injected with very small doses of HSA (0.1 μg) primed for a secondary response although antibody was not detected after one intravenous injection. The prolonged 17S antibody synthesis has not been reported for other hapten systems using protein carriers. Onoue et al (1968), however, found that rabbits given 2 intravenous injections of azaonaphthalene sulfonate groups coupled onto Salmonella typhimurium produced relatively high amounts of IgM antibody specific for the hapten. In addition, Kim and Karush (1973) elicited anti-lactose IgM antibody in rabbits by injection of a special strain of Streptococcus faecalis.

In contrast to the response obtained by intravenous injections, the response of birds given a single intramuscular injection of DNP-BGG in FCA was characterized by the exclusive and persistent synthesis of 7S antibody. Both 7S and 17S antibodies were detected, however, early in the immune response, i.e., within 14 days, but a month after immunization only 7S antibody was found. Chickens given a second injection of antigen in FCA at this time, yielded only 7S antibody. The PHA titers both during the primary and secondary responses were low ($\leq$ 1280) in contrast to the maximum titer of 10,240 found in chickens given 2 intravenous injections. Chickens injected with doses of antigen ranging from 0.02 to 20 mg in FCA yielded only 7S antibodies and these were produced for at least 490 days. When chickens were boosted by an intravenous

injection 493 days after priming only 7S antibodies were produced. Thus, even after a prolonged period, 17S antibody could not be stimulated by intravenous injection in chickens which had been primed with antigen in FCA. Since all chickens responded to 0.02 mg of antigen in FCA, the minimum dose required for a primary response was less than the dose needed when antigen was given intravenously.

The immune response of chickens given DNP-BGG in FCA appears to be similar to that of rabbits. Steiner and Eisen (1967b) found that rabbits injected with DNP-BGG in FCA produced IgM antibodies early in the immune response but later only IgG antibodies were found. They also observed that rabbits boosted with antigen in FCA produced exclusively 7S antibody. Werblin et al (1973b) measured the concentration of anti-DNP antibody in rabbits by equilibrium dialysis for a year after a single injection of 0.05 to 50 mg of antigen in FCA. In all cases, antibody was found for at least a year. Maximum amounts of antibody generally were obtained about 6 weeks after immunization.

In order to determine whether or not Mycobacterium in FCA was required for the continued and exclusive production of 7S antibody, chickens were injected with antigen in FIA. Both 7S and 17S antibodies were present 9 and 14 days after immunization, but by 20 days only 7S antibodies were synthesized and these persisted for at least 4 months. Thus, the responses of birds given FIA was qualitatively like that of birds injected with FCA. However, a quantitative difference between chickens given FCA when compared with those given FIA might exist. White and his colleagues (White 1970; Steinberg et al 1970; French et al 1970) examined the response of chickens injected with HSA incorporated

with various adjuvants. When the antigen binding capacity, as measured by the Farr method, was measured during the first 3 weeks no enhancement over control values was found by using depot-type adjuvants (aluminum phosphate, FIA), adjuvants with surface active properties (quarternary ammonium compounds, vitamin A), lipopolysaccharides, Bordetella pertussis or Corynebacterium parvum. However, they described a "biphasic" response which occurred in birds immunized with FCA and to a lesser extent in chickens given FIA. The amount of antibody in the first phase was similar to that obtained after a single intravenous injection of protein in aqueous solution. The response reached a peak at 8 to 12 days and decreased to a low level by 18 days. Later, a prolonged second rise of antibody occurred which peaked between 6 to 9 weeks. In the present study the amounts of anti-DNP antibody were not assessed throughout the immune response in chickens given adjuvants. It would be of particular interest to determine the concentrations of class of antibody that is synthesized since detectable levels of both 7S and 17S antibodies were found within the first 3 weeks. The initial phase might be due to 7S and 17S antibody followed by a progressive increase in 7S antibody production or affinity or both. Mond et al (1974) also found quantitative differences in the response of rabbits immunized with FCA versus those given FIA. Higher amounts of precipitating anti-DNP antibody were found in rabbits injected with DNP-BGG in FCA than in those injected with antigen in FIA. Despite the lack of quantitation, the data in the present study clearly indicate differences between the immune response of birds given intravenous injection and those given adjuvants in terms of the class of antibody formed.

That immunization with antigen in FCA affects the class or subclass of antibody synthesized was observed by others (Barth et al 1965; Coe 1966; Wilkinson & White 1966; Nussenzweig et al 1968; Warner et al 1968). Yet the mechanism for the stimulation of particular cell populations is unknown. One explanation might involve the affinities of the antibodies. Injection of low doses of antigen in FCA in chickens resulted in the production of antibodies of high affinities ($-\Delta F_1^\circ$ = 10.8 to 12.3 kcal/mole) by 17 weeks. The nonlinearity of Sips plots indicated that a shift in the distribution of antibody affinities had occurred due to the production of high affinity antibodies. Based on heterogeneity indices, the high affinity antibodies comprised a very restricted population. Similar results were obtained when chickens were given antigen in FIA. The 7S antibodies from 4 chickens were of high affinity ($-\Delta F_1^\circ$ > 10 kcal/mole) 13 weeks after immunization and the Sips plots of the binding data from 3 of the 4 antibody samples deviated from linearity. In contrast, chickens given intravenous injections of DNP-BGG failed to produce high affinity antibodies. Different priming doses (0.02 to 20 mg) were tried and various intervals between boosting (from 1 to 8 weeks) were used. Despite these differing protocols, the $-\Delta F$ values were less than 10 kcal/mole. The Sips plots were approximately linear in all cases. When 2 animals were tested immediately before boosting (16 weeks after the fourth injection) and after boosting (1 week and 3 weeks after the fifth injection), the 7S antibodies still had affinities less than 10 kcal/mole.

These results are in contrast with those reported by Mond et al (1974). They found that rabbits given 2 intravenous injections of

1.0 mg DNP-keyhole limpet hemocyanin 1 week apart and bled 14 days later produced antibody with a $-\Delta F_1^\circ$ value of 9.7 kcal/mole as determined by the Farr method. When these animals were boosted again on the 35th day and bled 7 days later, a $-\Delta F_1^\circ$ value of 10.8 kcal/mole was obtained for the antibody. On the other hand, when animals were primed with 15 mg of antigen and boosted with 5 mg 4 and 7 days later, antibody with very low affinity ($-\Delta F_1^\circ$ = 5.98 kcal/mole) was produced. In the present study, the effect of multiple injections of high doses of antigen was not tested. Perhaps antibodies of lower affinities might have been obtained. However, regardless of the priming dose, antibodies of high K_o were not stimulated. The discrepancy in the results might be accounted for by the differences in animal species, the protein carrier, the immunization protocol and the techniques used for determining binding affinities. Mond et al (1974) concluded that the major factor in determining the K_o of the antibody is cell selection by antigen. However, it is clear that other factors might be important in chickens. The intramuscular injection of antigen appears to be critical for the synthesis of 7S antibodies of high affinity. The fact that adjuvants might be required for optimal maturation of antibody affinity had been suggested earlier (Eisen 1966; Siskind & Benacerraf 1969). Few systematic studies have been done to test this hypothesis although Rodkey and Freeman (1970) found that BSA incorporated into FCA did not affect the avidity of the antibody produced.

It is possible that the factors which promote maturation of antibody affinity and those which result in the production of exclusive and persistent 7S antibody synthesis might be related. Thus the

synthesis of 7S antibodies is augmented by the fact that 7S antibody-forming precursor cells have high affinity receptors and are able to be continually stimulated by limiting concentrations of antigens. These factors are not the same, however, since when antigen is given in high concentrations (20 mg) in FCA high affinity antibodies were not formed yet only 7S antibody was produced. When chickens were given intravenous injections of antigen, maturation of 7S antibody affinities did not occur under the experimental conditions attempted and the 7S antibody was not produced exclusively. The molar ratio of 7S to 17S antibody was always greater than 1 but the 17S antibody-forming precursor cells were able to compete successfully with the 7S antibody-forming cell.

There are many possible mechanisms of action of adjuvants since there are a wide variety of substances which possess adjuvant activity and they may act in different ways. Some of the possibilities pertinent to the present study are summarized here but a more detailed description is found in two recent books (Jolles & Paraf 1973; Wolstenholme & Knight 1973). First, antigen emulsified with the adjuvant constitutes a depot thus allowing for the slow release of antigen into the circulation. This provides a source of continual antigenic stimulation. Perhaps the experiments of Feldbush and Gowans (1971) are relevant (see section II, B, 1). They found that if cells were allowed to reside in an antigen-free environment maturation of antibody affinity did not occur. This is probably an extreme situation. Antigen persists long enough without adjuvants to induce an immune response. The fact that chickens given multiple intravenous injections did not elicit the exclusive production of 7S antibody also argues against the theory that

the depot effect of adjuvants is the sole reason for adjuvanticity. In addition, Freund and Lipton (1955) showed that removal of the local site of injection as early as half an hour after stimulation, did not inhibit further antibody synthesis and the development of delayed hypersensitivity. The adjuvant and the antigen also can be injected in two different sites without loss in antibody responses (Merritt & Johnson 1963; Golub & Weigle 1967; Dresser 1968). Nevertheless, the persistence of antigen might be a contributing factor to the production of high affinity 7S antibody.

A second way in which adjuvants may exert their action is by the formation of a granuloma. A granuloma is composed of epithelioid cells, macrophages, and lymphocytes attracted to the site of injection. A strong immune response might depend on the relationship between the number of cells and the concentration of antigen which are optimal within a granuloma. It is possible that the formation of a granuloma is more important in chickens than in other animals since chickens do not possess organized lymph nodes. The spleen is the main peripheral lymphoid organ although diffuse lymphoid tissue is present throughout the body, as in the liver, lung, skin, caecum (Payne 1971). White and his colleagues found (White 1970; French et al 1970) that most of the antibody synthesized early in the immune response, i.e., in the first phase (see above), occurred in the red pulp of the spleen. Later, in the second phase, antibody was produced at the site of injection since intense plasma cell infiltration developed in the granuloma and specific antibody could be extracted from the granuloma in high yields when compated with other tissues. By analogy, perhaps a similar situation

occurred when chickens were injected with DNP-BGG. Intramuscular injection of antigen with adjuvants resulted in a granuloma where antibody was synthesized. Intravenous injection, on the other hand, stimulated antibody primarily from splenic cells. Perhaps there is a qualitative difference between cells of the spleen and those that migrate and make up the granuloma. For example, Steinberg et al (1970) suggested the antigen-sensitive cells in the spleen of the chicken convert to antibody-producing cells without forming the long-lived memory cells. However, injection of FIA resulted in a minimal granuloma formation and yet high K_o 7S antibody was produced.

Adjuvants might also act by enhancing the proliferation or differentiation or both of the cells involved in the immune response, i.e., the macrophages, B-cells and T-cells. Adjuvants might affect macrophage functions such as presentation of antigen, release of inflammatory factors, and control of the traffic of cells in lymphoid organs (Unanue 1972). Certain adjuvants appear to be selective B-cell mitogens, such as lipopolysaccharide and poly A:U. It is believed that any procedure that enhances B-cell proliferation allows for the more rapid selection of high-affinity antibody-producing cells by low concentrations of antigen (Gershon & Paul 1971; Siskind 1973). In chickens, FCA may affect B-cells (Tam & Benedict 1974). Injection of FCA emulsified with saline resulted in 2-fold increases in the level of "non-specific" 7S Ig, as determined by radial immunodiffusion. These immunoglobulins were not directed to the Mycobacterium in the adjuvant. Chickens immunized with FIA in saline or BSA incorporated in FIA did not synthesize demonstrable increases in 7S Ig (Tam & Benedict 1974).

Finally, adjuvants may act on T-cells. Three experiments have shown that T-cells might influence antibody affinity. Gershon and Paul (1971) found that adult thymectomized, lethally-irradiated, bone marrow-reconstituted mice produced less antibody of lower affinity than controls when injected with DNP-BSA. Reconstitution with 1 X 10^8 T-cells almost completely restored the immune capacity of these mice. Thymectomized mice immunized with DNP-KLH, however, still elicited considerable amounts of antibody but the affinity and the amount were lower than control non-thymectomized mice. A low number of T-cells (0.33 X 10^8) was sufficient to fully restore the response to control levels. The difference between the two immunogens may be due to a difference in the number of carrier-specific receptor cells in the nonimmunized T-lymphocyte population. Furthermore, Elfenbein et al (1973) found that antibody affinity was augmented by allogeneic lymphoid cells. Strain 2 guinea pigs were immunized with DNP-protein intraperitoneally on 3 successive days. Allogeneic cells from strain 13 guinea pigs which had been treated with FCA emulsified in saline were injected into primed recipients. Six days later the recipients were challenged. When compared with controls which did not receive cells, the allogeneic recipients produced slightly greater amounts of antibodies with a higher affinity (about 3-fold greater). A predominance of pfc's eliciting high avidity antibodies was observed as determined by hapten inhibition of indirect plaques.

Taniguchi and Tada (1974) attempted to define the role of T-cells on antibody affinity in rabbits. Procedural differences between this work and other studies should be noted. Adult thymectomy of rabbits

does not effect the immune response as profoundly as thymectomy of mice (Harris et al 1948; Good et al 1962; Archer et al 1962). Furthermore, rabbits were injected with 0.5 mg of DNP-BGG in FCA at monthly intervals throughout the period of study. Normal adult rabbits generally yielded relatively low amounts of anti-DNP antibody (2 mg/ml) as determined by the Farr test or by quantitative precipitin reaction and the antibody had only moderate affinity (maximum of 7×10^6 M^{-1}) as determined by equilibrium dialysis after specific purification. Also, the affinity of the antibody decreased from 30 to 150 days in normal rabbits immunized in this fashion. They postulated that the antigen may provide the selective pressure for B-cells but that such cells do not proliferate and differentiate into antibody-forming cells without the direct participation of helper T-cells. If enough T-cells are present, antibody affinity increases but if too many T-cells exist, the B-cells become "inactivated" and those selected by antigen with high affinity receptors are affected most easily. They suggested that this might account for the decrease in affinity that occurred long after immunization in their study and in those observed by others (Doria 1972; Kimball 1972; Urbain et al 1972; Ghose & Karush 1973; Werblin et al 1973b). Evidence for their hypothesis derives from the following observations: (1) Adult thymectomy or treatment with anti-thymocyte sera increased the amount (6 mg/ml) and enhanced the affinity ($K_o = 4 \times 10^7$ M^{-1}) of anti-DNP antibody produced in treated rabbits. (2) Rabbits preimmunized with optimal doses of carrier BGG in FCA stimulated large numbers of carrier specific T-cells but these suppressed the amount and affinity of the antibody. (3) Some rabbits made partially tolerant with large doses

of BGG given intravenously before immunization elicited low amounts of antibody with low affinity. These conditions apparently inhibited the helper functions of T-cells which, in turn, prevented B-cell activity.

It is apparent that T-cells may act either by enhancing B-cell activity resulting in a more efficient selection of high affinity cells or by suppressing B-cell activity. The factors which dictate whether augmentation or suppression of the immune response occurs require better definition. Based on this discussion, ascribing the most important factor in the regulation of antibody class and affinity is not possible. The original suggestion that the antigen concentration (Eisen & Siskind 1964; Eisen 1966; Steiner & Eisen 1967b; Siskind & Benacerraf 1969) affects antibody affinity was confirmed in chickens injected with varying doses of DNP-BGG in FCA. However, this does not seem to be the sole reason for maturation of antibody affinity; if it were, a comparable maturation would be expected for birds given antigen in saline. Adjuvants might exert their action by the slow release of antigen, by the formation of granuloma or by affecting the activities of macrophages, T-cells or B-cells. The fact that the administration of antigen in FIA appears to be as effective for the selection process as FCA on a qualitative level must also be considered. A delineation of the mechanisms of action of adjuvant activity requires the use of less complex adjuvants and the testing of their effects on pure cell populations either by reconstitution in vivo or in tissue culture.

Comparisons between the amounts of antibody produced in birds given antigen in FCA and those given intravenous injections were not possible by the methods used in this study because of the increase of non-specific

immunoglobulins which occurs in birds given FCA (see above). However, comparisons were made among birds given intravenous injections at various intervals and using different priming doses. It was found that more antibody was produced when the interval between injections was short (1 week) than when the interval was longer (3 to 8 weeks). Also, less antibody was produced in birds given very low (0.02 mg) or very high (20 mg) doses of priming antigen than with intermediate doses (0.2 or 2.0 mg) when a 1-week interval between injections was used.

Several investigators have suggested that two types of memory cells might exist: a short-termed and a long-lived memory cell (Nossal 1962; Celada 1967; Feldbush 1973). Perhaps intravenous injections elicited primarily short-lived memory cells. The fact that less antibody was produced in birds given very low doses can be explained simply by assuming that insufficient antigen was given to elicit optimal amounts of antibody. With very high doses of priming antigen, partial tolerance may have been induced. Theis and Siskind (1968) induced partial neonatal tolerance in rabbits by injections of large doses of antigen. The rabbits, when injected with antigen in FCA, produced antibodies of very low affinity when compared with controls. They postulated that tolerance preferentially affected high affinity cells. In the present study, however, no striking differences in affinity were observed in chickens given 20 mg of antigen although the concentration of antibody decreased 2-fold. Recent studies (Heller & Siskind 1973; Weksler et al 1973) on adult rabbits gave a relationship compatible to these results. Adult rabbits rendered partially tolerant by an intravenous injection of 40 mg of DNP-BGG, had suppressed amounts of antibody but the antibody

affinity was only slightly decreased when compared with controls. Perhaps the tolerance induced in adults is different than that found in neonates.

A further complicating factor is the possible presence of circulating antibody at the time of the booster injection. It is known that the passive administration of antibody can specifically suppress the subsequent immune response (Werblin & Siskind 1972a; Siskind 1973). In addition it was found that the higher the affinity of the passive antibody, the less was required for suppressing antibody synthesis (Walker & Siskind 1968). Antibody appears to compete for the available antigen. Also, partially suppressed animals elicit residual antibody of higher affinity than do controls, demonstrating that only cells with very high binding receptors are stimulated. The presence of circulating antibody has a dual role: it suppresses the immune response by making less antigen available for stimulation and it enhances selection since only very high binding cells will proliferate. In chickens given intravenous injection, it is doubtful that the circulating antibody had a significant effect on the results. If it were involved, it would be expected that a lower concentration of antibody of higher affinity would be produced at the 1-week week interval when circulating antibody could be detected after priming than at the 8-week interval when antibody was not present. Instead, more antibody was found at the shorter interval and no consistent difference in the K_o values was observed.

Considerable controversy exists concerning the comparison between IgM and IgG affinity and the existence of IgM maturation. Reports relating to these questions were reviewed above (see section II, B, 1).

Recently, Kim and Karush (1974) published an elegant study on the properties of anti-lactose antibodies in a horse. Specifically purified IgM and 7S antibody were prepared from samples taken from the 6th to the 14th week after the start of immunization. Each was fractionated by liquid isoelectric focusing. The major component in the IgM population exhibited a maximum affinity of 4×10^5 M^{-1} which was observed throughout the 8-week period of study. They suggested that early maturation of this IgM fraction may have occurred before testing. Another fraction in the IgM population showed about a 2-fold increase but it did not reach as high an affinity as the major component (2.7×10^5 M^{-1}). On the other hand, all of the 7S antibody fractions showed 4 to 10-fold increases in antibody affinity and all reached a common maximum value of 2×10^6 M^{-1}. The fact that the maximum affinity of the IgM antibody was lower than the 7S antibody indicates that these do not share V-regions. They suggested that this finding did not favor the concept of "IgM to 7S switch" during the immune response.

In the present study nearly indentical affinities and heterogeneity indices were obtained for the 7S and 17S antibodies isolated from 12 individual chickens given 2 intravenous injections of antigen. Both antibody populations appeared to be normally distributed with a similar range of affinities. Despite the similarity in affinities, the 7S and 17S antibodies may have different variable region amino acid sequences. Other evidences, however, indicate that the 7S antibody-forming cell may have derived from the 17S antibody-forming cell during ontogeny (see section II, B, 3). If this were the case, then there must be very high affinity ($-\Delta F^\circ$ > 10 kcal/mole) 17S antibody-forming cells from

which the high affinity 7S antibody-producing cells were generated. Intramuscular injections of antigen with adjuvants stimulated the high affinity 7S antibody-producing cells but either failed to induce or shut off 17S antibody synthesis. Thus the conditions required to generate circulating IgM antibody of high affinity are not known. Perhaps high affinity 17S antibody was synthesized but it was removed from the circulation rapidly as an antigen-antibody complex because of its greater functional affinity for the antigen, or as suggested by Kim and Karush (1974) perhaps the high affinity IgM-producing cells were used selectively for the "switch." Anternatively, if there is no relationship between 7S and 17S Ig producing cells, some mutational process might act selectively on 7S antibody-forming cells to generate antibody of high affinity. However, the postulate that both 7S and 17S antibody-forming cells have similar binding properties but that the regulation of their expression differs is favored.

Valences less than 2 were obtained for 7S antibodies of low affinity whereas a valence close to 2 was found for antibody of high affinity. Similarly, 17S antibody of low affinity gave Scatchard plots which extrapolated to values less than 10, the valence expected based on structural considerations. Although these results do not directly show that IgM antibody had 10 functional binding sites as suggested by others (see section II, B, 1) the theory that each IgM molecule consists of 5 strong and 5 weak sites appears to be untenable. There is no reason to suppose that 17S antibody is less heterogeneous than 7S antibody since both 7S and 17S antibodies showed similar heterogeneity indices. Thus,

even if each molecule were composed of 5 strong and 5 weak sites this would not be observed because heterogeneity would create a curvature of the Scatchard plot. The fact that valences less than 2 could be generated from 7S antibody preparation by contamination with non-binding proteins or by the presence of antibody of very low affinity indicates that these parameters most easily explain valences less than 10 for 17S anti-hapten antibodies obtained by others.

The conclusions reached in the present study were dependent on the validity of the techniques used. Radioimmunoelectrophoresis was employed as a rapid and direct method for determining the presence or absence of 7S and 17S antibodies. However, the sensitivity of RIE depends on many parameters. Experimental conditions must be optimal, i.e., sufficient labeled antigen and potent developing anti-globulin antiserum that precipitates strongly with both 7S and 17S immunoglobulins are needed. The IgA-like antibody was not detected in any of the preparations. Specific antibody may have been present but perhaps the rabbit anti-globulin sera did not develop the IgA-like immunoglobulin. Lower amounts of 17S antibodies might be detected than 7S antibodies because of the higher valence of the 17S antibody. Also, the test is more sensitive for antibodies of high affinity than for those of lower affinity. As observed in other animal species, some chicken pre-immune sera bound DNP-proteins weakly by RIE. Both the 7S and 17S Ig, and tentatively an α-2 macroglobulin were identified as the proteins which bound DNP. It is not known what leads to their production. Some appear to be anti-bodies since after immunization sera with preimmune activity in some

cases (Table VI, chickens 1250 and 1253) showed more intense activity than sera without preimmune binding. Perhaps antibodies cross-reacting to DNP may have been induced by exposure to other antigen.

The concentration of antibody binding sites in the globulin preparations was determined directly from equilibrium dialysis measurements by extrapolation from 1/B versus 1/C plot. This value was used to construct the Sips plot, log $1/(B_t - B)$ versus log C. In this way K_o was determined without requiring the specific purification of antibody. This technique might select for a population of antibody molecules. In fact, the K_o of the specifically purified preparations from chickens given intravenous injections were consistently lower ($-\Delta F^\circ < 7.5$ kcal/mole) regardless of the valence used for determining the affinity than those obtained from globulin preparations. In addition, inaccurate extrapolation from the Scatchard plots resulted in considerable alteration in the K_o value (Figure 9). Slight errors also might occur when the method described by Werblin and Siskind (1972b) is used, but the effect on K_o is small. When studying heterogeneous low affinity populations that exhibited considerable curvature of the 1/B versus 1/C plot, the total concentration of the binding sites (B_t) tended to be underestimated. In addition the presence of small amounts of high affinity antibody in a population which consisted primarily of low affinity antibodies resulted in an underestimate of B_t (Kim et al 1974). When B_t is underestimated, "a" is underestimated but K_o is overestimated. Thus, the actual $-\Delta F^\circ$ values for those birds given intravenous injections might be slightly lower by about 0.3 kcal/mole than the experimental values. The general conclusions of this study are not affected by this

consideration. In fact, the actual K_o might give more convincing evidence that intravenous injections of antigen did not elicit antibodies of high affinity.

In this study, the concentration of antibody as determined by equilibrium dialysis measurements was expressed on the basis of either 10 mg 7S or 1 mg 17S globulin (Figure 17) instead of the usual per ml of whole serum. Earlier determinations of the amounts of anti-DNP by this method (Werblin & Siskind 1972b) required the assumption that the globulin preparations consisted solely of IgG antibody and that the final globulin amount obtained was equivalent to that found in whole serum. In the studies of chickens given intravenous injections, both 7S and 17S antibodies were found. Isolation of the 2 immunoglobulins was necessary. This resulted in considerable procedural losses during repeated gel filtration, and in the case of the 17S antibody, there was additional loss because of the passage through a rabbit anti-7S Ig Fc immunoadsorbent column. The concentrations of 7S and 17S Ig in adult chickens are about 7.5 and 0.7 mg/ml whole serum, respectively (Table I). Thus, if the concentration of antibody in 1 ml of whole serum were to be calculated on the basis of 10 mg 7S Ig and 1 mg 17S Ig per ml of serum, then the concentrations of antibody would appear to be overestimated. However, crude globulin preparations were used, and Ouchterlony analyses showed that these preparations contained other proteins besides the immunoglobulins. In addition, this procedure assumed that these chickens have roughly the same amount of 7S and 17S globulin except for the negligible variation in antibody levels. Nevertheless, the method is valid for comparative purposes. Despite the possible errors in the

calculations of antibody concentrations in whole serum, affinity determination was dependent only on the accuracy of the antibody concentration in the globulin preparations. Although this method is cumbersome, it is a primary test which does not depend on secondary (precipitation, agglutination, complement fixation) or tertiary (in vivo) immune reactions which often fail to measure considerable amounts of antibody, nor does it depend on the affinity of the antibody. Thus, the advantages appear to outweigh its limitations.

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