University of Glasgow

CONTROL OF THE QUANTITY AND QUALITY OF ANTIBODY BIOSYNTHESIS IN THE DOMESTIC FOWL

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SUMMARY

It has been assumed that the factors which control antibody biosynthesis in the chicken, and their mode of operation, are subsequently reflected in the characteristics of the antibody response. These characteristics were taken to include the kinetics of antibody biosynthesis, as well as the quantity, the quality (i.e. the affinity, avidity and heterogeneity) and the class of the antibody molecules themselves. This assumption is particularly justifiable in the chicken, where the relative short half life values of immunoglobulins ensure that the characteristics of the antibody response reflect preceding events in the induction and control of biosynthesis fairly rapidly and with reasonable accuracy.

One of the most important factors which exert control on the biosynthesis of antibodies is the immunogenicity of the antigen and the influence of immunogenicity on the processes of cell selection and induction of biosynthesis of antibodies in chickens was examined. For example, the effect of antigen structure upon the production of specific antibody in chickens was studied - this involved investigation of the proficiency of different carriers and the effect of epitope density upon the antibody response to the DNP hapten. Another consideration is the presentation of the antigen, and this entailed examination of the effect of carrier pre-immunisation upon the response to a hapten coupled to that carrier and also investigation into the action of adjuvants. Although birds of all ages produced detectable amounts of antibody when immunised with a protein antigen such as HSA, and the kinetics of the

response and the avidity of the antibodies could be readily determined, the age of chickens did appear to have a considerable bearing upon their ability to produce anti-DNP antibodies. The kinetics of the anti-DNP response were very similar to the pattern observed for production of anti-HSA antibodies; however, the affinity of chicken anti-DNP antibodies, as measured by equilibrium dialysis, was found to be very low and not affected significantly by the dose of antigen.

The effect of adjuvants upon biosynthesis of antibodies in chickens was also investigated. From the characteristic kinetics of antibody biosynthesis following immunisation with antigen in Freund's complete adjuvant, as well as from the pattern of antibody avidity, the distribution of antibody classes and the effect of adjuvant on homeostasis of the antibody response in chickens, certain conclusions concerning the mode of action of adjuvants were reached; their influence either on the selection process or on the regulation of biosynthesis was discussed.

Efficient regulation of antibody production in chickens was readily inferred from the kinetics of biosynthesis. This homeostatic mechanism was studied by analogy with the effects of passive antibody upon the production of specific antibodies, and the possibility that regulation of biosynthesis was achieved by antibody-mediated feedback inhibition was discussed.

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SUMMARY

It has been assumed that the factors which control antibody biosynthesis in the chicken, and their mode of operation, are subsequently reflected in the characteristics of the antibody response. These characteristics were taken to include the kinetics of antibody biosynthesis, as well as the quantity, the quality (i.e. the affinity, avidity and heterogeneity) and the class of the antibody molecules themselves. This assumption is particularly justifiable in the chicken, where the relative short half life values of immunoglobulins ensure that the characteristics of the antibody response reflect preceding events in the induction and control of biosynthesis fairly rapidly and with reasonable accuracy.

One of the most important factors which exert control on the biosynthesis of antibodies is the immunogenicity of the antigen and the influence of immunogenicity on the processes of cell selection and induction of biosynthesis of antibodies in chickens was examined. For example, the effect of antigen structure upon the production of specific antibody in chickens was studied - this involved investigation of the proficiency of different carriers and the effect of epitope density upon the antibody response to the DNP hapten. Another consideration is the presentation of the antigen, and this entailed examination of the effect of carrier pre-immunisation upon the response to a hapten coupled to that carrier and also investigation into the action of adjuvants. Although birds of all ages produced detectable amounts of antibody when immunised with a protein antigen such as HSA, and the kinetics of the

response and the avidity of the antibodies could be readily determined, the age of chickens did appear to have a considerable bearing upon their ability to produce anti-DNP antibodies. The kinetics of the anti-DNP response were very similar to the pattern observed for production of anti-HSA antibodies; however, the affinity of chicken anti-DNP antibodies, as measured by equilibrium dialysis, was found to be very low and not affected significantly by the dose of antigen.

The effect of adjuvants upon biosynthesis of antibodies in chickens was also investigated. From the characteristic kinetics of antibody biosynthesis following immunisation with antigen in Freund's complete adjuvant, as well as from the pattern of antibody avidity, the distribution of antibody classes and the effect of adjuvant on homeostasis of the antibody response in chickens, certain conclusions concerning the mode of action of adjuvants were reached; their influence either on the selection process or on the regulation of biosynthesis was discussed.

Efficient regulation of antibody production in chickens was readily inferred from the kinetics of biosynthesis. This homeostatic mechanism was studied by analogy with the effects of passive antibody upon the production of specific antibodies, and the possibility that regulation of biosynthesis was achieved by antibody-mediated feedback inhibition was discussed.

ABBREVIATIONS AND SYMBOLS

a	index of heterogeneity of antibody affinity, identical to the parameter c used by Sips (1948).
ABC_{30}	antigen-binding capacity: a primary measure of the amount of antibody in an antiserum in terms of the serum dilution at which 30% of added antigen at a specified total concentration is bound by antibody; from this the amount of antigen which would bind to 1 ml of undiluted antiserum is calculated.
Ab	antibody.
Ab_t	total concentration of antibody combining sites, measured as the total antigen-binding capacity of a unit volume of antiserum at high concentrations of free antigen.
AFC	antibody-forming cell.
AFPC	antibody-forming precursor cell.
Ag	antigen.
B cell	bursa-derived lymphocyte or its functional equivalent in mammals.
BGG	bovine gamma globulin.
BSA	bovine serum albumin.
C_H	constant region of the immunoglobulin heavy chain.
C_L	constant region of the immunoglobulin light chain.
cm	centimetre.
cpm	counts per minute.
CSA	chicken serum albumin.
DEAE	diethylaminoethyl.
DNA	deoxyribonucleic acid.
DNFB	2,4-dinitrofluorobenzene.
DNP	dinitrophenyl.

DNP_x	conjugated dinitrophenyl groups; x = number of substituent groups per carrier molecule.
EACA	ε-amino-n-caproic acid.
E_{280}	spectrophotometric extinction or absorbance (= $\log_{10}$ Io/I, where Io is the incident intensity and I is the transmitted intensity) of a solution at wavelength denoted by subscript in nm.
$E_{280}^{1\%}$	spectrophotometric extinction or absorbance of a 1% solution (10 mg ml^{-1}) at wavelength denoted by subscript in nm.
ε_{280}	spectrophotometric absorbance of a 1 molar solution at wavelength denoted by subscript (i.e. molar extinction coefficient; mol^{-1} l. cm^{-1}).
EDTA	ethylenediaminetetra-acetic acid.
e.g.	exempli gratia (for example).
et al.	et alii (and others).
etc.	et cetera (and the rest).
et seq.	et sequentia (and what follows).
$F(ab')_2$	divalent fragment (containing two antibody combining sites) obtained by digestion of immunoglobulin with pepsin.
Fc	crystallizable fragment obtained by digestion of immunoglobulin with papain.
FCA	Freund's complete adjuvant.
FIA	Freund's incomplete adjuvant.
g	acceleration due to gravity at the earth's surface (= 9.81 m s^{-2}); centrifugal force is expressed as a multiple of this value.
g	gram.
H	hapten molecule(s).

^{3}H	tritium.
HBC_{30}	hapten-binding capacity: a primary measure of the amount of antibody in an antiserum in terms of the serum dilution at which 30% of added hapten at a specified total concentration is bound by antibody; from this the amount of hapten which would bind to 1 ml of undiluted antiserum is calculated.
HCN	haemocyanin.
HGG	human gamma globulin.
HSA	human serum albumin.
HuRBC	human red blood cells.
i.e.	id est (that is).
IgA	immunoglobulin A.
IgG	immunoglobulin G.
IgM	immunoglobulin M.
im	intramuscular.
ip	intraperitoneal.
iv	intravenous.
*I	radioactive iodine (i.e. ^{131}I or ^{125}I).
kcal	kilocalorie.
kg	kilogram.
K_o	average affinity or mean intrinsic association constant of anti-hapten antibodies (litres $mole^{-1}$).
K_R	average avidity or relative association constant of antibodies.
κ	kappa light chain of an immunoglobulin molecule.
l.	litre.
LPS	lipopolysaccharide.

λ	lambda light chain of an immunoglobulin molecule.
λmax	wavelength of maximum spectrophotometric absorbance of a solution (in nm).
M	molarity (moles litre^{-1}).
mA	milliampère.
mCi	millicurie.
mg	milligram.
ml	millilitre.
mol	mole.
MW	molecular weight (daltons).
µg	microgram.
µl	microlitre.
µm	micrometre.
ng	nanogram.
nm	nanometre.
nmol	nanomole.
oz	ounce.
OVA	ovalbumin.
P	probability of the difference between two means of two samples occurring as a result of random sampling errors.
PBS	phosphate ($H_2PO_4^-/HPO_4^{2-}$, 0.01M, pH 7.2) buffered saline (0.15M NaCl).
PCA	passive cutaneous anaphylaxis.
PFC	plaque-forming cell.
R_{av}	average radius of centrifuge rotor, measured from the rotor centre to half-way down the sample tube.
rpm	revolutions per minute.
S	Svedberg unit (= 10^{-13} seconds) for expressing sedimentation constants.

SE	standard error.
SRBC	sheep red blood cells.
Σ	operator denoting summation over all experimental points.
$T_{\frac{1}{2}}$	half-life time.
T cell	thymus-derived lymphocyte.
TCA	trichloroacetic acid.
V	volt.
V_H	variable region of the immunoglobulin heavy chain.
V_L	variable region of the immunoglobulin light chain.
v/v	proportion, volume/volume, expressed as a percentage or as a ratio.
w/v	proportion, weight/volume, expressed as a percentage.

PHYSICO-CHEMICAL CONSTANTS

The following values for physico-chemical constants were used in this thesis:

	Molecular weight (daltons)	Spectrophotometry
Human serum albumin	69,000	$E_{280}^{1\%} = 5.8$
Ovalbumin	46,000	$E_{280}^{1\%} = 7.35$
Immunoglobulin G	150,000	$E_{280}^{1\%} = 13.0$
ε-DNP-lysine	312	$\varepsilon_{360} = 17{,}400$ $mol^{-1}\ l.cm^{-1}$
ε-DNP-aminocaproic acid	297	$\varepsilon_{365} = 17{,}800$ $mol^{-1}\ l.cm^{-1}$

DEFINITION OF CERTAIN IMMUNOCHEMICAL TERMS USED IN THIS THESIS

Because of the necessity for accurate statements in describing immunochemical concepts, the following terms, which will be used frequently in this thesis, have been interpreted thus:

AFFINITY is a thermodynamic expression of the strength of binding between the antibody combining site and an antigenic determinant and thus of the stereochemical compatibility between them, i.e. it is a measure of complementarity whereby the antibody combining site and an antigenic determinant can come into sufficient proximity to maximise the effects of chemical bonding. As such, affinity may be measured by the equilibrium or association constant (K litres $mole^{-1}$) for the antigen-antibody interaction, but since there is most usually heterogeneity of affinities within a population of antibody molecules of defined specificity, it is at the best an average value referred to as the "geometric mean intrinsic association constant," (K_o litres $mole^{-1}$). Affinity is most accurately applied to interactions involving simple, uniform determinants, e.g. haptens, thus obviating the difficulty of having to consider heterogeneous determinants on the same molecule.

AVIDITY is an expression used to describe the strength of binding between antibody and a complex antigen. Because the antigenic entity has a number of heterogeneous determinants, avidity is a composite description of the overall antibody-antigen interaction; it is however somewhat more complicated than a simple summation of affinities for individual determinants, and because of the effective multivalency of the antigen there is often a co-operative "bonus" effect.

Various definitions of avidity have been and are used, and to a large extent these definitions, as well as the quantitation of avidity, can only be expressed in terms of the particular technique (e.g. binding of radio-labelled antigen, inhibition of plaque formation, inhibition of phage neutralisation) used to measure avidity and usually require that a number of fundamental assumptions are made.

For example, in this thesis avidity has been measured as K_R, the geometric mean association constant obtained from the Sipsian version of the equilibrium equation for an antigen antibody interaction - assuming that each antigen molecule of a homogeneous population interacts with only one antibody combining site.

INTRODUCTION

Immunity is the ability of an individual to recognise as foreign any material or substance which has penetrated the physical barriers of the body and which does not belong to the native constitution of that individual; the term refers especially to the variety of responses (usually specific but including non-specific mechanisms) by which the body subsequently attempts to eliminate the invading entity. A specific immune response consists of the production of antibody immunoglobulins and/or specifically reactive cells, both of which are circulated in the body and interact physically with the antigen; this interaction will potentially result in the inactivation, death or phagocytosis of the invading material. These two types of immune response are respectively that of humoral immunity and that of cell-mediated immunity. The experimental work in this thesis deals explicitly with humoral immunity in chickens and the factors controlling this response.

Antibody molecules are the executives of the humoral immune defences and in fulfilling this role they constitute a highly discriminating molecular recognition system. When an animal is subjected to an infection or is inoculated with antigen artificially, the ensuing immune reaction has two fundamental properties. First of all there is the actual responsiveness of the individual to consider: thus in most cases, but not all, antigenic stimulation provokes some sort of antibody response and the animal incurs some memory of this stimulation. The second feature of antibody biosynthesis is its potential diversity such that the antibody response to any one antigen involves exclusive production of molecules of that specificity and even within a given specificity there is usually considerable heterogeneity of antibody affinities.

The formation of antibodies is governed by several control mechanisms. This much is apparent from observation of the antibody responses induced in laboratory animals: not only are the amount and duration of antibody production (i.e. the responsiveness) subject to finite limits, but the antibody molecules themselves are restricted to a single specificity complementary to the antigen involved - indicative of the potentially enormous diversity of antibody formation. Obviously, elucidation of the control of antibody production both in terms of its inducibility and its diversity is important in our understanding of immunity against infection and tumour antigens, as well as in the management of allergies, auto-immune disease and in tissue transplantation.

It was the object of this thesis to obtain information about the mechanisms which control antibody biosynthesis in the domestic fowl; the experimental approach was to study the characteristics of the antibody response (i.e. the concentration, kinetics, affinity, heterogeneity and specificity of antibodies) and to discover the way in which these properties were influenced by the nature of antigenic stimulation (the type, dose, presentation and schedule of administration of antigen), as well as by the presence of passive antibody and by the operation of negative feed-back homeostasis.

THE SCOPE OF CONTROL MECHANISMS AFFECTING ANTIBODY BIOSYNTHESIS

The Induction of Antibody Biosynthesis

In the preceding short description of how humoral immunity is controlled, it was stated that there are two fundamental properties of the antibody response which are subject to control; the first point to be considered was the induction of antibody biosynthesis. This refers to whether or not a certain antigen is recognised by the individual as being foreign, and then whether it either induces active production of antibodies, or stimulates cell-mediated immunity, or both. When an antigen is encountered by the immune defences of an individual, there are at least four recognised effects which can occur. Firstly, lymphocytes may be triggered to differentiate and proliferate to become either antibody-forming cells or the specialised cells which participate in cell-mediated immunity. The second possibility is the induction of complete or partial unresponsiveness to the antigen in the form of specific immunological tolerance. A third alternative is that neither induction of biosynthesis nor induction of tolerance take place, for example if there is insufficient antigen or else the antigen structure is non-immunogenic. In addition there is often the establishment of immunological memory, whereby the specificity of an antigen encountered for the first time is remembered by the immune system and a second encounter with the same antigen results in enhanced responsiveness (or unresponsiveness). These effects are by no means all mutually exclusive and they can probably occur concurrently in a variety of combinations.

The inducibility of an antibody response (i.e. the particular combination of the above effects which is ultimately observed) depends on a number of factors, both intrinsic and extrinsic. The intrinsic factors (which have not been investigated but which must be mentioned) may include for example, the genetic control exerted by the histocompatibility-linked immune response (Ir) genes, positive and negative feed-back mechanisms mediated by specific antibody, the synergism of different cell populations, and the rate of catabolism of the antigen. The extrinsic factors which control the induction of antibody biosynthesis depend on the nature of the antigenic stimulus: thus the quantity, complexity, chemical structure, route and rate of administration of antigen as well as the use of adjuvants all contribute to determine the eventual characteristics of an immune response. These variable aspects of antigenic stimulation were considered to be particularly relevant for studying control processes governing the induction of antibody biosynthesis.

The Diversity of Antibody Biosynthesis

The other property of humoral immunity is the diversity of antibody production; i.e. each individual is capable of producing an enormous number of different specificities of antibody and within the confines of a given specificity there is very often considerable heterogeneity of the antibody population. In more precise terms the diversity of the antibody combining sites is likely to be particularly informative because it is probably a strict reflection of the immunoglobulin receptors selected by antigen during the induction of the immune response.

Immunoglobulin molecules have a genetically determined primary sequence of amino acids, which in turn determines the secondary and tertiary structures and hence the steric configuration of the antibody combining site. Direct evidence for this comes from experiments on the reversible denaturation of antibodies, where specificity is lost upon denaturing the molecule but is faithfully restored by renaturation (Freedman and Sela, 1966).

It seems logical that differences in specificity are a result of variations in the primary amino acid sequence and the physical participation of the variable regions of both heavy (V_H) and light (V_L) chains in providing contact amino acids within the combining site has been confirmed by the results of affinity labelling experiments whereby a suitably identifiable hapten was inserted into the combining site and then remained in association with the amino acids involved following digestion of the immunoglobulin. Such experiments (e.g. Singer and Doolittle, 1966) have shown that three main segments in the light chain and possibly four segments in the heavy chain contribute contact amino acids within the active site and it was found that these segments correlated well with the hypervariable regions identified by statistical analyses of immunoglobulin primary sequences (Koshland, 1966). They also appeared in relatively similar positions in the variable parts of both heavy and light chains, indicating a symmetrical arrangement of the two chains around the active site. The structure of immunoglobulin molecules is therefore genetically pre-determined by the genome of the individual and is not subject to extrinsic controlling influences.

Although the structure of each immunoglobulin molecule resides ultimately in the cellular DNA, the process by which a certain configuration (or set of configurations) of the antibody combining site may be multiplied and proliferated is controlled by certain extrinsic factors. A selective theory, whereby the antigen selects the complementary specificity from a pre-existing range rather than giving instructions for a completely innovatory structure to be synthesised was first proposed by Ehrlich (1900) and later reiterated and elaborated (Jerne, 1955; Burnet, 1959; Lederberg, 1959; Siskind and Benacerraf, 1969) as a working model for antibody biosynthesis. The clonal selection theory formulated by Burnet (1959) has now become one of the central principles of immunology and briefly this hypothesis proposes that at some time in ontogeny, and independently of antigen, an individual either inherits or acquires the potential to respond uniquely to any one of an enormous range of antigens. Therefore the operation of clonal selection would require that the individual possessed a complete repertoire of genetic information (a body of germ line V genes probably provides the requisite diversity of antibody molecules; somatic mutation could augment this variation further, but there is as yet no firm evidence for this), such that there was the potential capacity to produce specific antibodies capable of counteracting each and every antigenic contingency. It is envisaged that this state of preparation takes the form of clones of pre-committed antigen-reactive precursor cells, i.e. each lymphocyte becomes committed to the expression of one V_L and one V_H gene which combine with the C region genes to provide a template for antibody of given specificity, class and type. Given that these

clones are produced, the selection process can only be achieved if there is some means of trapping, localising or effectively concentrating the antigen at the surface of receptive precursor cells. This is followed by antigen-driven selection of the appropriate antibody specificity; each precursor lymphocyte (or clone) is responsive to one, or at the most a restricted number of similar antigenic determinants. This commitment is mediated by antigen-specific receptors on the cell surface, and these receptors will have antigen-binding characteristics similar to those of the eventual antibodies secreted. Then, once the precursor cells of the appropriate specificity have been selected, biosynthesis moves into the inductive phase. It is now widely accepted that the register of genetic information relating to different antibody specificities is effectively indexed by antibody representatives on the surface of precursor antibody-forming cells. Indeed, it is difficult to visualise how the selection process could function without some kind of specific receptors. In this way, an antigen is both recognised as being foreign and will subsequently provoke an immune reaction, by virtue of the fact that there are already some complementary structures in existence within the individual. It is proposed that the antigen associates preferentially with those precursor cells bearing receptors whose stereochemical configuration most suitably accommodates the determinant antigenic structure (i.e. of highest affinity for the antigen). This concept of complementarity is clearly not an all or nothing idea and the average affinity and the range of affinities for a response would be expected to depend, amongst other things, upon antigen

concentration. This point is illustrated in Figure 1; at low antigen concentrations only those receptors of most exact complementarity (i.e. where the free energy of association between receptor and antigen is greatest), will be selected. Higher antigen concentrations will involve, in addition, less perfect but nonetheless satisfactory combinations.

The control of specificity is therefore apparent at both the molecular and cellular levels. Refined genetic control determines the stereochemical configuration of individual molecules; thereafter, those cells bearing receptors having the highest affinity for the antigen are preferentially stimulated to proliferate and to differentiate (or become tolerised). Accordingly, the requirement of clonal selection for :- (1) Precursor cells of predetermined specificity, was manifested by the demonstration that non-immune animals possessed small numbers of lymphoid cells which could interact specifically (presumably by means of membrane receptors) with antigen, e.g. haemocyanin (Byrt and Ada, 1969), BSA (Naor and Sulitzeanu, 1967) or DNP (Davie and Paul, 1971) and that these apparently pre-committed cells could be removed by affinity chromatography (Wigzell and Andersson, 1969a) or by "killing" with lethally radioactive antigen (Ada and Byrt, 1969). (2) Receptors on these precursor cells of which the specificity (Wigzell and Andersson, 1969a; 1969b; Mäkelä, Cross and Ruoslahti, 1969; Ada and Byrt, 1969), the avidity (Andersson, 1972; Davie and Paul, 1970; 1972a; Julius and Herzenberg, 1974), and the type of heavy chain (Walters and Wigzell, 1970) were identical to those of the eventual secreted

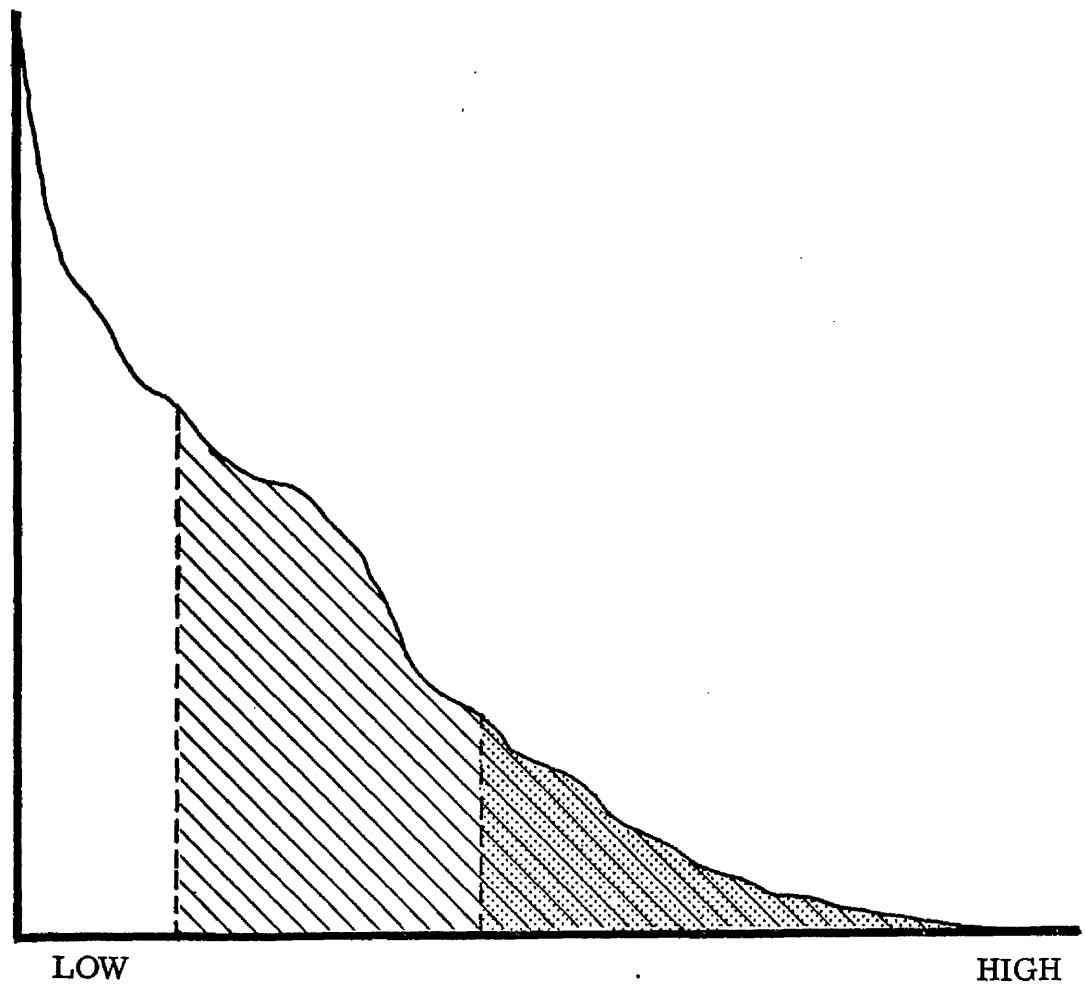

FIG. 1. Hypothetical distribution of lymphocytes with respect to the affinity of their receptors for a given antigen — to illustrate how the affinity of antibody formed may be expected to depend on antigen dose.

antibody molecules, has also been shown experimentally. The prediction of cell selection that a single plasma cell subsequently produces one type of molecule exclusively has been convincingly confirmed by experimental evidence: e.g. single antibody-producing cells in microdroplets caused immobilisation of organisms belonging to one or other Salmonella serotype, but not both (Nossal and Lederberg, 1958); also, immunofluorescence has been used to demonstrate that plasma cells from rabbits immunised with two different antigens could be stained for antibody to one or other of the antigens, but not both (White, 1958), or that cells from an animal immunised with a hapten on a protein carrier could be stained either for hapten or for carrier specificity but not both (Green, Vassalli, Nussenzweig and Benacerraf, 1967). In addition, immuno-enzymological techniques have similarly shown monospecific cells in animals immunised with alkaline phosphatase and peroxidase (Avrameas, Taudou and Ternynck, 1971). In the heterozygous rabbit double immunofluorescent staining for two allotypes controlled by allelic genes has shown the existence of two plasma cell populations - one population containing one allotype and the other the alternative one (Pernis, Chiappino, Kelus and Gell, 1965); finally, there is good evidence that a plasma cell tumour will only produce one unique type of myeloma protein molecule (Awdeh, Williamson and Askonas, 1970).

MANIFESTATIONS OF CONTROL MECHANISMS IN OPERATION

A productive phase of specific antibody biosynthesis may be characterised according to the following criteria, and these may be regarded as a reflection of the control of both the inducibility and

the diversity of the response.

1. The absolute quantity of antibody produced or in the circulation at a given time after immunisation.

2. The quality of antibody, in terms of its primary binding properties (i.e. affinity or avidity).

3. The heterogeneity of antibodies within a population: both idiotypic variation manifested in the distribution of binding affinities and also the proportional representation by different isotypes.

The Quantity of Antibody

The quantity of antibody produced by chickens following immunisation is dependent upon the type, dose and presentation of the antigen, and also upon the genetic constitution of individual and different strains of birds. After immunisation with a single injection of soluble protein, specific antibody has rarely been shown in the circulation before four days, but thereafter the levels of antibody have been found to rise - reaching peak values at approximately the seventh day (Benedict, Brown and Hersh, 1963; White, French and Stark, 1970). Subsequently there was an exponential decline in the antigen-binding capacity of the serum (White, 1973). A similar pattern has been observed in haemagglutinin and splenic plaque assays of antibody production in response to immunisation with SRBC, although the earliest detection and attainment of peak levels of specific antibody were fractionally advanced in time (White, 1973). The primary response of the chicken to antigens like HSA or SRBC is

characteristically different from that found in mammals, especially in the later phases of IgG* antibody production. Mammalian production of IgG antibody may be described in two phases; an early phase in which the IgG antibody shows an initial rapid rise to reach a maximum level after about 8 days. The second phase comprises a very gradual rise in IgG antibody titres for 2 - 3 weeks, to reach a plateau which may be maintained for several weeks or even months (e.g. Uhr, Finkelstein and Baumann, 1962). In contrast, the chicken shows a steady decline in serum antibody levels after the peak titre has been reached at about seven days after immunisation. That this decline phase of the avian response reflects a total cessation rather than a gradual diminution of antibody biosynthesis was inferred from the fact that the rate of regression of the antigen-binding capacity of serum antibody was virtually identical to the rate of catabolism of chicken IgG in the body (White, 1973); i.e. the decline of antibody levels in the serum had the same half-life as passively injected radiolabelled chicken IgG. (There have in fact been conflicting values reported for the half-life times of chicken antibodies; White (1973) has given a value of 1.93 days, whereas Wostman and Olson (1969) have found a value of 4 days for the half-life time, rising to 8 days in germ-free chickens).

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*Chicken 7S immunoglobulin is sometimes called "IgY" because of its lack of identity with gammaglobulins from other vertebrates (Mehta, Reichlin and Tomasi, 1972). However, it will be referred to forthwith as IgG, in keeping with common practice.

Although the production of anti-hapten antibodies by the chicken has been described (Gold and Benedict, 1962; Orlans, Saunders and Rose, 1968; Orlans, 1968; Gallagher and Voss, 1969; Voss and Eisen, 1972; Sarvas, Mäkelä, Toivanen and Toivanen, 1974; Yamaga and Benedict, 1975a) and the antibodies have been characterised (Orlans, 1968; Gallagher and Voss, 1969; Voss and Eisen, 1972; Yamaga and Benedict, 1975a; 1975b), several authors have noted how surprisingly difficult it is to elicit biosynthesis of large amounts of anti-hapten antibodies in chickens either by attention to the mode of immunisation (Orlans, Saunders and Rose, 1969) or to the primary dose and the frequency of immunisation (Yamaga and Benedict, 1975a). Certainly there has been a lack of systematic examination of the kinetics of anti-hapten antibody production in chickens.

The Quality of Antibody

The quality of antibody is governed primarily by the fact that during the processes of antigen recognition, antigen (or at least a determinant part of it) will associate preferentially with those immunocompetent cells which carry surface receptors of sufficient affinity to bind the antigen (in this respect therefore, affinity is a marker of the V region of the immunoglobulin molecule and hence of the genes coding for it). This means that those membrane receptors of high affinity for the antigen will compete with more success for the antigen than cells bearing low affinity receptors. Hence only cells with the higher affinity receptors will be stimulated as antigen becomes increasingly scarce by virtue of catabolism or removal by antibody. As a means of defence it is in the best

interests of the individual to have circulating antibodies of the highest possible affinity, and indeed the direct relationship between antibody affinity and its biological activity has been shown; e.g. Siskind and Eisen (1965) found that the amount of purified anti-DNP antibody required to give a positive PCA reaction in rabbits became less as the association constant of the antibody increased; also, Fauci, Frank and Johnson (1970) demonstrated that complement fixation by antigen-antibody complexes increased as the affinity of IgG antibody for its antigenic determinant became greater. On the other hand, Soothill and Steward (1971) have shown that variations in the antibody affinity of individuals may be of pathological significance in that mice prone to nephritis (following neonatal infection with lymphocytic choriomeningitis virus) produced antibody of lower affinity to certain soluble protein antigens compared with nephritis-resistant mice. Antibody affinity would appear to be subject to indigenous genetic control, since it has been found that affinity varies between different strains of rat (Lamelin and Paul, 1971) or mouse (Petty, Steward and Soothill, 1972; Steward and Petty, 1976) or for different rabbit allotypes (Werblin, Kim, Mage, Benacerraf and Siskind, 1973).

On the basis of a cell selection theory Eisen (1966), and Siskind and Benacerraf (1969) have reasoned that the average affinity of antibodies will increase with time following immunisation. Indeed, both the avidity (Jerne, 1951; Talmage and Maurer, 1953; Farr, 1958; Sarvas and Makela, 1970) and antibody affinity (Eisen and Siskind, 1964; Fujio and Karush, 1966; Klinman, Rockey,

Frauenberger and Karush, 1966; Little and Eisen, 1966; Zimmering, Lieberman and Erlanger, 1967; Parker, Godt and Johnson, 1967; Goidl, Paul, Siskind and Benacerraf, 1968; Siskind, Dunn and Walker, 1968; Andersson, 1970; Harel, Ben-Efraim and Liacopoulos, 1970; Lamelin and Paul, 1971) have been found to increase with time by a variety of experimental approaches; this "maturation" pattern was also apparent in respect of the average receptor affinity for radio-labelled antigen of a population of lymph node cells from immune guinea pigs (Davie and Paul, 1972a). Further experiments by Davie and Paul (1972b) have shown that the maturation of antibody affinity reflected the maintainance of high affinity antibody-producing cells accompanied by a gradual loss of the low affinity producers rather than a sequential appearance of progressively higher affinity clones. In contrast it has recently been reported by Yamaga and Benedict (1975a) that there was no appreciable rise in the average affinity of chicken anti-DNP antibodies (either 7S or macroglobulin) following immunisation with a single or with repeated injections of antigen. However, in experiments with mammals where the maturation of antibody affinity has been well documented, it was found that the rate of maturation of antibody affinity, at least above a minimal level of antigenic stimulation, was inversely related to the original dose of antigen. Thus the rate of maturation of antibody affinity was slower when larger doses of antigen were used (Eisen and Siskind, 1964); in other words the continued recruitment of low affinity antibody-producing cells was less with lower concentrations of antigen. In the same way it was found that the maturation of anti-DNP affinity was inversely related in a non-linear fashion to the number of DNP groups on the carrier molecule

(i.e. the DNP:carrier molar ratio) used for immunisation (Larralde and Janof, 1972; Klaus and Cross, 1974; Quijada, Kim, Siskind and Ovary, 1974). Further evidence has been provided by the demonstration that memory cells were eliminated by passage down an affinity chromatography column less efficiently if they were taken at an early stage rather than later on during the course of the antibody response (Wigzell and Andersson, 1969a).

It has been suggested that as the affinity of an antibody for a certain determinant increases, then its affinity for similar determinants also rises - until the point is reached where that antibody becomes cross-reactive. In support of this idea Little and Eisen (1969) found that as the affinity of anti-DNP antibody increased, so the affinity of that antibody for the picryl group (TNP) rose. The tendency for an antiserum to become more cross-reactive (to "degenerate") with time after immunisation led Gershon and Kondo (1972) to suggest that the antiserum was in fact becoming more avid.

The observed rise in antibody affinity with time could be rationalised in two ways: firstly, that the relatively large amounts of antigen which are present directly after immunisation interact preferentially with circulating antibody of high affinity - resulting in its selective removal in the early stages of a response. Secondly, it may be true that there is a time-dependent shift in cell populations, from one synthesising antibody of low average affinity towards a population producing antibody of high average affinity. The latter alternative has been found to hold, at least <u>in vitro</u>; thus, in an

antigen-free cell culture system the affinity of antibody produced during a brief incubation period was directly related to the prior duration of the antibody response in the animals from which the cells for culture were obtained (Steiner and Eisen, 1967).

More recently it has been suggested that high and low affinity antibodies may alternate during the course of biosynthesis (Macario and De Macario, 1973) or that variations in antibody affinity may parallel the changes in antibody titres (Urbain, Van Acker, De Vos-Cloetens and Urbain-Vansanten, 1972). It has also been found that the mean association constant tended to decrease towards the end of an antibody response (Doria, Schiaffini, Garavini and Mancini, 1972; Urbain et al., 1972) and that low affinity antibody was still present during the decline phase of the response (Macario and De Macario, 1974). In addition, there is good evidence that antibodies of low affinity do in fact persist for a considerable length of time, concurrent with a rise in the proportion of high affinity antibodies (Werblin and Siskind, 1972a; 1972b) and that such persistent low affinity antibody could be re-activated by a second stimulation with the antigen (Möller, Bullock and Makela, 1973). One possible explanation for this is that these persistent low affinity antibodies were in some way connected with the process of memory (Macario and De Macario, 1974). The variable patterns for the maturation of antibody affinity described above could be explained by a fluctuating predominance of antibody-forming cell clones; thus when a dominant clone declined to a low level of antibody biosynthesis, another clone could then be stimulated and subsequently take over the

task of producing antibody (Williamson and Askonas, 1972; Askonas and Williamson, 1972). Moreover, if serum antibodies were sampled at any time during these periods of clonal flux they would be considerably heterogeneous (and therefore probably of variable average affinity), being a mixture of products from cell clones in either their ascendancy or their decline. It would seem then that the consecutive changes in antibody affinity which have been observed are not as simple as was first believed, and that the pattern perhaps reflects more complex processes than the straightforward selection of cells by antigen. Although the effect of clonal selection upon antibody affinity is very relevant, it probably constitutes a contributory rather than an exclusive influence in determining the dynamics of clonal replacement and the effect this has on the affinity of antibodies.

The investigations into antibody affinity described above usually dealt with entire antibody populations and therefore predominantly with IgG antibodies. Early work on the binding properties of IgM antibodies showed no significant maturation of IgM affinity with increasing time after immunisation (Sarvas and Mäkelä, 1970; Baker, Prescott, Stashak and Amsbaugh, 1971; Huchet and Feldmann, 1973) and that they were always of low affinity anyway relative to IgG antibodies (Mäkelä, Ruoslahti and Seppälä, 1970). However, recent advances in techniques for detecting antibody secretion at the cellular level and for studying antibody binding at the cell population level, have furnished contrary evidence. For example, by studying the relative binding characteristics of IgM plaque-forming

cells secreting anti-hapten antibodies using the plaque inhibition technique, it was found that the concentration of free hapten required to give 50% reduction in IgM PFC numbers decreased with time after immunisation - indicating that an increase in average binding affinity of PFC antibodies had occurred (Wu and Cinader, 1972; Claflin, Merchant and Inman, 1973). Antibodies of the IgM class specific for the Forssman antigen on sheep erythrocytes became increasingly avid (assessed by the ease with which antibody could be dissociated from sheep erythrocytes) with time after immunisation (Linscott, 1969). In the chicken however, Yamaga and Benedict (1975a) have reported that anti-DNP macroglobulin antibody, elicited by intravenous administration of antigen, was of a low affinity which did not increase either with the passage of time or with repeated immunisation.

Whether or not the affinity of IgM antibodies shows maturation characteristics comparable to those of IgG antibodies, it is generally true to say that IgG antibodies predominate within the high affinity antibody population, and that in most cases the affinity of IgM antibody is low relative to that of IgG. Such a situation may be explicable in terms of a controlled switching from IgM to IgG production. On the assumption that IgM receptors are pentameric, cells carrying these receptors would bind antigen at multiple sites on the cell surface (e.g. possibly 10 combining sites per receptor), thereby allowing IgM cells of relatively low affinity to be triggered, whereas from the IgG cells only those of higher affinity could be stimulated. This would be directly due to the valency of IgM,

meaning that the "functional affinity" of the whole IgM molecule represented a total amplification of the mean intrinsic association constant for each combining site by a factor as high as 10^6 (Hornick and Karush, 1972). It has been argued by Mäkelä, Kostiainen, Koponen and Ruoslahti (1967) on this basis that intact antigens may stimulate a considerable number of IgM-producing cells and some IgG-producing cells; then, as the antigen is degraded and catabolised, there is no longer such advantage in having multivalent receptors and the antigen fragments will associate more and more with IgG receptors of higher affinity. This argument is favoured by the empirical finding that large antigens of uniformly repeating structure characteristically induced biosynthesis predominantly of IgM antibodies (Möller, 1965; Britton and Möller, 1968); the hypothesis is further supported by the demonstration that a densely haptenated carrier elicited proportionately more IgM production than its lightly haptenated counterpart (Kontiainen, 1971; Sarvas and Mäkelä, 1974). This sort of hypothesis for an antigen-driven switch, which proposes a shift in cell populations from those producing IgM antibodies to cells producing IgG antibody of increasing affinity, is challenged by arguments that such a switch is genetically controlled, and it has been postulated that this may begin within the non-specific environment in which stem cells are induced to become B lymphocytes, before any clonal selection is accomplished by antigens (Cooper, Lawton and Kincade, 1972).

It is clear then that a process of clonal selection based purely on receptor affinity is an over-simplification, since it takes

no account of the differences between cells producing IgM and those producing IgG antibodies, nor does it consider the switching of cells from IgM to IgG production, or the importance of the anatomy of lymphoid systems for concentrating foreign materials around the cells which can respond to them. Other mechanisms which may also influence the quantity and affinity of antibody are possible interactions among different cell populations, the best studied example being the co-operation between B and T lymphocytes. The presence of two distinct populations of lymphocytes (B cells derived from the bursa of Fabricius in chickens and probably from the bone marrow in mammals, T cells derived from the thymus in both cases)and their collaboration in bringing about antibody biosynthesis was first shown by Claman, Chaperon and Triplett (1966) who noted the lack of antibody production when pure populations of either thymocytes or bone marrow lymphocytes were transferred into different irradiated host animals and challenged, and contrasted this with the successful biosynthesis of antibody when a mixed population of lymphocytes was similarly tested. The cells responsible for secretion of antibodies were shown in chickens to be bursa-derived (Cooper, Peterson, South and Good, 1966) and in mammals to be B lymphocytes (Mitchell and Miller, 1968). The role of T lymphocytes as "helper cells" in an anti-hapten antibody response was demonstrated by Mitchison (1969) and Rajewsky (1971) who both found that primed B cells made a secondary antibody response to a hapten bound to carrier only when T cells primed to the carrier were present. In other words the T cells recognise and respond to carrier determinants and in so doing they somehow promote the development of certain B lymphocytes into cells producing antibody to the hapten. A variety of mechanisms have been proposed to explain co-operation,

and these include: (1) The antigen is presented in a multivalent form following congregation of antigen molecules as an ordered array on the carrier-specific T cell (for instance promoting low affinity B cell receptors to form multiple cross-links of high total affinity). (2) A chemical factor (i.e. a T lymphokine) is secreted which acts as a second signal on B cells independently of antigen; e.g. co-operation has been demonstrated between T and B cells when the two populations were separated on either side of a porous membrane (Feldmann and Basten, 1972). (3) There exists some form of amplification mechanism whereby T cells specific for the antigen secrete cytophilic receptors which can then endow other cells, including possibly macrophages, with the same specificity and the same ability to co-operate with B cells; e.g. Feldmann (1972a) has shown that macrophages are required before an in vitro response to a thymus-dependent antigen could be evoked. Although the precise mechanism of co-operation is not yet clear, the importance of the phenomenon in the induction of antibody biosynthesis has been demonstrated convincingly; e.g. (1) Cells synthesising anti-hapten antibody of high affinity were shown to derive from the co-operative effects between B cells and helper anti-carrier T cells (Schirrmacher, 1971). (2) Thymectomy of mice caused a decrease in the amount of anti-hapten antibodies which the animals could subsequently produce (Taylor, 1971). (3) In the absence of T cells, anti-hapten antibody affinity was reduced (Gershon and Paul, 1971). (4) Thymus-independent antigens have been found to favour the production of macroglobulin antibodies (Ada, Nossal, Pye and Abbot, 1963).

The Heterogeneity of Antibody

The heterogeneity of antibodies provides another source of information about control mechanisms affecting antibody production; in this respect, heterogeneity includes the differences between isotypes as well as the heterogeneity of idiotypes within a given antibody class.

Considering first of all the relative proportions of different antibody classes in an immune response, the chicken has been found to produce demonstrable amounts of IgM antibodies during the early phase of a primary response to both soluble and particulate antigens (Benedict, Brown and Hersh, 1963). This early production of IgM antibodies rapidly gave way to biosynthesis of IgG, which predominated at later stages. Recent work by Yamaga and Benedict (1975a) implied that intravenous immunisation of chickens with repeated doses of DNP-BGG led to biosynthesis of both 7S and macroglobulin anti-hapten antibodies but that both classes were produced only in small amounts.

It has been proposed that cells which start out as producers of IgM are subsequently switched to producing IgG antibodies; Nossal, Szenberg, Ada and Austin (1964) reached this conclusion from the observation that the number of cells producing mercaptoethanol-sensitive antibodies declined during the course of an immune response. In the chicken, treatment with anti-IgM of embryos which were subsequently bursectomised at hatching caused total suppression of IgM production as well as partial suppression of IgG production, suggesting that

inhibition of IgM biosynthesis hindered the development of IgG-producing cells (Kincade, Lawton, Bockman and Cooper, 1970). In addition, it was found by Pernis, Forni and Amante (1971) that a small proportion of rabbit peripheral blood lymphocytes which were actively synthesising IgG nonetheless carried an IgM marker on their surface membranes, implying that a transition from IgM to IgG production had occurred. Kincade and Cooper (1971) provided further evidence that this transition took place in individual cells rather than reflecting a gross shift in antibody-producing cell populations; thus, by using different fluorescent antibodies to chicken μ or γ chains to detect the appearance of antibody classes in single cell suspensions, a high proportion of single bursal cells containing heavy chains of both IgM and IgG were demonstrated. The presence of immunoglobulin receptors in the surface membranes of a proportion of unstimulated B lymphocytes, either from the central lymphoid organs or from the peripheral circulation, has been confirmed using specific fluorescent antibodies (Rabellino, Colon, Grey and Unanue, 1971). Furthermore, there is good evidence, obtained by acrylamide gel electrophoresis of radiolabelled heavy and light chains of immunoglobulins isolated from solubilised plasma membranes of splenic lymphocytes (Vitetta, Baur and Uhr, 1971; Marchalonis and Cone, 1973), that the receptor on lymphocyte membranes has the characteristics of a monomeric IgM molecule (often referred to as IgMs), even for those cells destined to produce IgG antibodies; it has been shown equally for those cells whose secretory product is pentameric IgM (Vitetta and Uhr, 1973). It must be pointed out that the finding of a

ubiquitous monomeric IgM receptor for all antigen-sensitive cells is at variance with the supposition put forward on page 20 , where the occurrence of pentameric IgM receptors was postulated. It seems therefore quite possible that a lymphocyte or immunoblast with surface IgM receptors switches to the biosynthesis of IgG and at the same time is transformed into a cell with the morphological and ultrastructural features of a plasma cell. This would preclude any requirement for surface IgG receptors, although it has been found that receptors of the IgG class were present on the surface of antigen-stimulated mouse spleen cells in vitro (Pierce, Solliday and Asofsky, 1972). Further evidence for the switch from IgM to IgG is seen in the low proportions of IgM in the antibody response upon secondary stimulation. In mammals there is usually a lesser proportion of IgM in the total antibody content of a secondary response compared with the proportion of IgM antibody in a primary response (e.g. Bauer, Mathies and Stavitsky, 1963); this could reflect a more rapid switch from IgM to IgG biosynthesis, or else the switch that has taken place during the primary response has been incorporated in the immunological memory. In chickens it has been reported that hyperimmune birds had no macroglobulin antibody in their serum (Dreesman and Benedict, 1965), although there is also evidence suggesting that the proportion of IgM antibody in a secondary response to particulate antigens (e.g. virus or bacteriophage) was increased relative to the primary response (Szenberg, Lind and Clarke, 1965).

The control of antibody biosynthesis is also reflected in idiotypic heterogeneity; i.e. the structural differences between

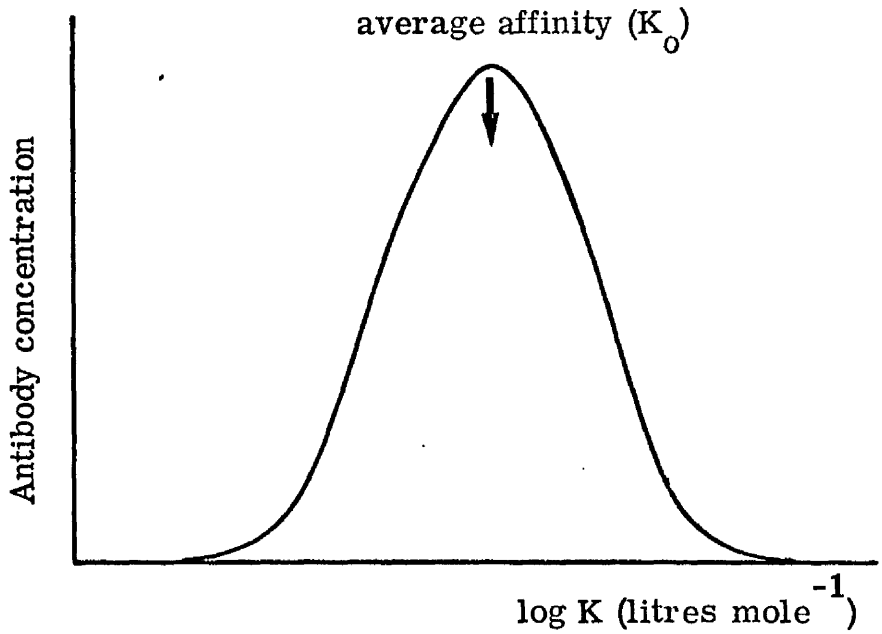
individual immunoglobulin molecules of the same specificity and class, found in any one animal. Even within the same specificity the number of different idiotypes is enormous; for example, Kreth and Williamson (1973) have estimated that the number of different molecules with antibody properties to a given hapten in immune inbred mice, was probably about 8000. A selective theory implies that for any given antigen, even if all its determinants are identical in structure, there will usually be a variety of receptors with different affinities for the determinants. This variety of individual antibody affinities will be reflected in the heterogeneity of the antiserum as a whole, and there is a considerable amount of evidence for such heterogeneity within antibody populations: e.g. (1) From the analysis of primary binding data (Pauling, Pressman and Grossberg, 1944; Karush, 1956; Nisonoff and Pressman, 1958). (2) From the iso-electric separability of various antibodies of the same specificity and class (Williamson and Askonas, 1972). (3) From the chemical fractionation of an antibody population (Schlossman and Kabat, 1962; Gelzer and Kabat, 1964; Eisen and Siskind, 1964). (4) From physical differences between individual combining sites (Warner and Schumaker, 1970; Carson and Metzger, 1974).

Information obtained from studying binding affinities was at first found to be consistent with the assumption of a continuous symmetrical distribution of antibody affinities (in terms of the free energy change), and this distribution could be adequately described in terms of either a Gaussian (Pauling, Pressman and Grossberg, 1944) or a Sipsian (Nisonoff and Pressman, 1958) distribution function. This

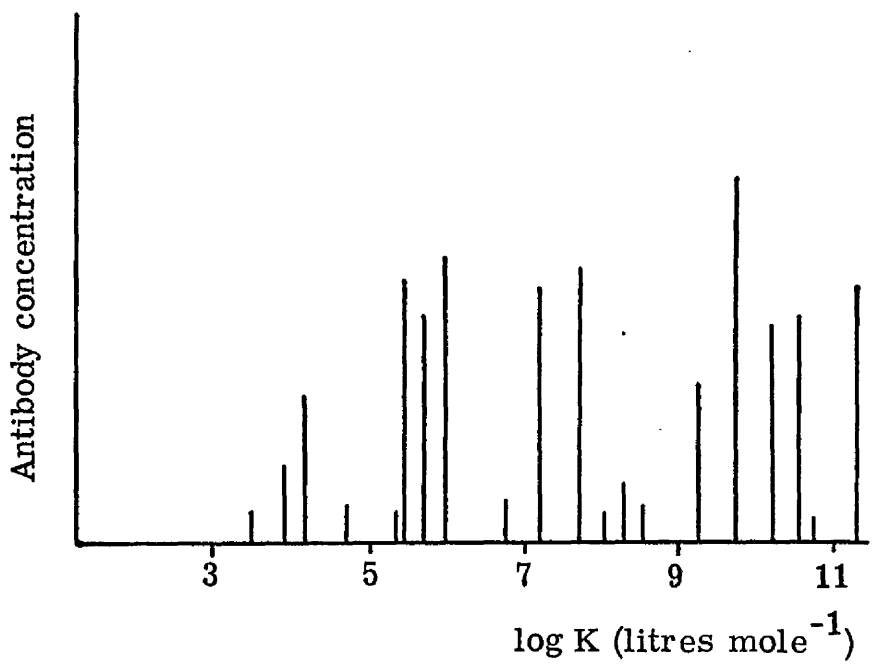
sort of assumption was convenient and useful as a first approximation of the geometric mean association constant, but more sophisticated analysis of antibody-hapten interactions has led to the belief that the distribution of affinities is by no means continuous and/or symmetrical in every case (Roholt, Grossberg and Pressman, 1968; Roholt and Pressman, 1968; Pressman, Roholt and Grossberg, 1970; Werblin and Siskind, 1972a; Werblin, Kim, Quagliata and Siskind, 1973; Kim and Siskind, 1974; Kim, Werblin and Siskind, 1974; Mukkur, Szewczuk and Schmidt, 1974). The development of a computational model involving an iterative approximation technique to account for any departures from the normal distribution (Werblin and Siskind, 1972a), has indicated that the distribution may in fact be skewed or even bimodal. Using this method to study the maturation of antibody affinity with time, the distribution of affinities was found to be symmetrical initially but subsequently became negatively skewed, i.e. with most of the antibody occurring towards the high affinity end of the distribution range (Werblin and Siskind, 1972a; Werblin, Kim, Quagliata and Siskind, 1973). As mentioned previously, there also appeared to be significant amounts of low affinity antibody which persisted throughout the course of biosynthesis (Werblin and Siskind, 1972b; Werblin et al., 1973). The sort of advances in assessing heterogeneity which have just been referred to have helped considerably to elucidate some of the principles underlying idiotypic heterogeneity. Moreover, the concept of discontinuous distributions of affinity could possibly explain the conflicting accounts of heterogeneity which have appeared in the past; e.g. an overall rise in heterogeneity with time has been reported (Eisen and Siskind, 1964; Miller and Segre, 1972),

or decreased heterogeneity, or indeed more complex patterns (Kitagawa, Grossberg, Yagi and Pressman, 1967; Roholt, Seon and Pressman, 1970; Jaton, Waterfield, Margolies, Bloch and Haber, 1971; Kimball, 1972; Macario, De Macario, Franceschi and Celada, 1972; Macario, De Macario and Celada, 1973; Claflin, Merchant and Inman, 1973). The concept of antibody heterogeneity being symmetrical and continuous is contrasted in diagrammatic form in Figure 2 with the more recent idea that heterogeneity of antibody affinities may be represented more accurately by a non-symmetrical discontinuous distribution.

It was originally thought that the heterogeneity of antibodies within a population was a direct reflection of the heterogeneous nature of the antigen; according to this theory, the heterogeneity of anti-hapten antibodies produced in response to immunisation with a hapten-carrier conjugate was due to the different environments in which otherwise uniform haptenic groups were situated on the carrier (Singer, 1964; Parker, Godt and Johnson, 1966). Control of antibody specificity solely by a process of selection would, on this basis, predict that a more homogeneous population of antibodies is produced in response to more homogeneous antigens. In concurrence with this hypothesis it has been found for example that a relatively well-defined DNP-polypeptide antigen of regularly repeating structure stimulated an antibody response of restricted heterogeneity (Richards and Haber, 1967; Haber, Richards, Spragg, Austen, Volloton and Page, 1967; Pappenheimer, Reed and Brown, 1968; Richards, Pincus, Bloch, Barnes and Haber, 1969). A similar result was found following



(a). Continuous, symmetrical distribution (of the form described by Pauling, Pressman and Grossberg, 1944).



(b). Discontinuous non-symmetrical distribution (of the form described by Werblin and Siskind, 1972a).

FIG. 2. Two different models of the heterogeneity of antibody affinities.

experimental immunisation either with a hapten coupled in a definite known position on a small carrier (Trump and Singer, 1970; Montgomery, Rockey and Williamson, 1972; Keck, Grossberg and Pressman, 1973) or with hapten conjugated to an autologous protein carrier (Escribano, 1974). It has also been reported that if a carrier-attached hapten was variously expanded by adding on other chemical groups (e.g. DNP with either glycylglycylglycine or with p-amino-benzolyl glutamate), and these antigens were used to immunise rabbits, then the temporal variations in the binding affinities of antibodies for the original hapten decreased as the site-filling capacity of the antigenic determinant increased (Woehler, Cannon, Clark and Lovins, 1974): these authors suggested that this effect was due to the restriction of the number of ways in which the determinant could orientate itself, as its size increased, within the combining site of a receptor. Immunisation of responder guinea-pigs with a series of α-DNP- and ε-DNP-oligolysines caused production of antibodies which "recognised" (as assessed by the maximum binding affinity) the exact chain length (± 1 lysine residue) of the oligolysine carrier molecule (Levin, Levine and Schlossman, 1971). Evidence of this sort therefore lends support to the idea that the carrier provides suitably immunogenic environments for haptenic determinants, and that idiotypic heterogeneity reflects the structural variations between the different sites of hapten substitution on the carrier. On the other hand however, there is a considerable amount of experimental evidence which argues against such a local environment hypothesis as the basic cause of idiotypic heterogeneity. For instance, the carrier molecule has been shown to contribute <u>per se</u> only very slightly towards the total energy of

interaction between a hapten-carrier conjugate and specific anti-hapten antibody (Eisen, 1964a; Schlossman, Ben-Efraim, Yaron and Sober, 1966). Similarly, antibody produced either in guinea-pigs against ε-29-DNP-insulin (Little and Counts, 1969), or in rabbits against ε-41-mono-DNP-ribonuclease (Eisen, Simms, Little and Steiner, 1964), had no apparent restriction in its range of anti-DNP affinities. Also, the "carrier effect" was still evident and maintained its specificity even when a long spacer molecule was inserted between the carrier and the hapten (Mitchison, 1967; Ungar -Waron, Gurari, Hurwitz and Sela, 1973). Even more confusing to the overall picture have been claims of limited heterogeneity or even complete homogeneity of anti-hapten antibodies elicited in response to immunisation with antigens of considerable heterogeneity, such as azo-p-benzoate-BGG (Nisonoff, Zappacosta and Jureziz, 1967; Roholt, Seon and Pressman, 1970). The alternative to the local environment hypothesis is that each individual possesses a large set of variable region genes and this results in intrinsic heterogeneity of the receptor combining sites suitable for any one determinant; Montgomery and Williamson (1972) have pointed out that there are probably discrepancies in the expression of these V-region genes between individuals of the same species and strain. The distribution which is eventually observed may well constitute the dual influences of both mechanisms; i.e. the effect of the local environment of hapten groups on the heterogeneity of antibodies and the result of inherent genetic variability of receptor combining sites. Finally, it is worth remarking how these different hypotheses regarding the control of antibody heterogeneity have influenced thinking in

devising experimental methods for producing antibodies of restricted heterogeneity: e.g. (1) Immunisation with a homogeneous antigen. (2) The cloning of antibody-producing cells. (3) By selection of individuals producing myeloma protein with the properties of specific antibody. (4) The chemical and physical fractionation of heterogeneous antibody populations.

The Induction of Tolerance

So far the scope of control mechanisms controlling antibody biosynthesis has included only the active production of specific antibodies, describing how the quantity, quality and heterogeneity of antibody is subject to control and how these parameters are affected. However it is equally a characteristic of the immune response that under certain circumstances a state of specific unresponsiveness is encountered, when an individual becomes tolerant to a certain antigen; this form of control may, for example, represent the way in which the distinction between self and non-self components is made.

Tolerance is a specific state of unresponsiveness resulting in partial or complete inability to respond to a certain immunogenic stimulus by virtue of prior (tolerising) contact with the antigen; it has been found applicable to particulate, soluble and haptenic antigens (Dixon and Maurer, 1955; Cinader and Dubert, 1956; Dresser, 1961; Boyden and Sorkin, 1962; Sercarz and Coons, 1963). The first possibility in trying to explain tolerance is that immunocompetent cells are eliminated; it has been suggested that this results either from irreversible inactivation (Dresser and Mitchison, 1968; Iványi

and Salerno, 1972) or from terminal differentiation (Šterzl, 1966). It is attractive however to contrast this with a model whereby the tolerogen remains on the surface of the antigen-binding cell instead of that clone of cells being deleted; i.e. the tolerant cell does exist, but its receptors have been blockaded in a reversible fashion. Autoradiographic studies in mice which had been made either immune or tolerant to DNP were done by reacting radio-labelled anti-DNP antibody with splenic lymphocytes, and these investigations showed that cell receptors maintained their specificity for DNP both in normal and tolerant animals; moreover, in tolerant animals it was concluded that tolerogen remains on the cell surface - whereas in immune animals the antigen does not (Aldo-Benson and Borel, 1974). By treating lymphoid cells with trypsin, either before or after being tolerised in vitro, Diener and Feldmann (1972) were able to prevent or abrogate respectively the state of specific unresponsiveness. It was also found that transfer of tolerised cells into irradiated recipients quickly restored the immunocompetence of the transferred cells (Sjöberg, 1972) - again suggesting that the tolerisation was reversible. This sort of reversible mechanism of receptor blockade would satisfy the requirements for a selective process in either the induction of tolerance or immunity, and could account for the events of acquired and self tolerance. In what manner the state of tolerance is influenced by cell co-operation is not fully understood, although claims for its origin in the population of helper cells (Rajewsky, 1971), or in the hapten-specific cell precursors (Feldmann, 1972b), or in both (Chiller, 1970) have been made. Another suggestion has been that high dose paralysis incapacitated both B and T cell function, whilst low dose paralysis affected the helper T cells only (Andersson, Celada and Åsjö, 1974).

One route of investigation into the mechanisms of tolerance has been the study of antibody affinity in partially tolerant animals, or in those which have recovered from or have "broken" tolerance. If cells have to compete for antigen during the induction of tolerance, then the high affinity receptor-bearing cells would be the first target for the tolerogen; thus any cells still able to produce antibody would have receptors of low affinity and would therefore produce antibody of low affinity. Depression of antibody affinity under such conditions has been reported (Theis and Siskind, 1968; Harel, Ben-Efraim and Liacopoulos, 1970; Urbain et al., 1972; Davie, Paul, Katz and Benacerraf, 1972). Conversely, it has also been claimed that induction of tolerance had no effect on the affinity of small amounts of antibody which were produced (Mitchison, 1964).

A once tolerant animal has often been found to regain its immunological capabilities with the passage of time; in such cases the affinity or avidity of antibody produced has apparently increased as the animal "escaped" from the tolerant state, until these parameters attained similar values to those found in conventionally immune animals (Theis, Thorbecke and Siskind, 1968; Dowden and Sercarz, 1968). Such a loss of tolerance could be explained in two ways relative to the different hypotheses for tolerance outlined above: (1) It represents a recruitment of virgin cells (Dresser and Mitchison, 1968). (2) It reflects the spontaneous recovery of paralysed (blockaded) cells (Dowden and Sercarz, 1968).

Tolerance may be broken by immunising the tolerant animal with an antigen which is a cross-reacting or a modified form of the tolerogen (Weigle, 1961; Paul, Siskind and Benacerraf, 1967; Iványi, 1972). However, antibody produced under these conditions most probably reflects the synthesis of a cross-reacting antibody of low affinity for the tolerogen. This has been confirmed to some extent by the finding that if the initial tolerising dose was sufficiently large to suppress even low affinity (and therefore potentially cross-reactive) antibody-producing cells, then it proved impossible to break tolerance (Paul, Thorbecke, Siskind and Benacerraf, 1969).

The exact mechanism of tolerance is still in doubt, although it obviously represents an aspect of control which affects the induction of antibody biosynthesis and which is highly antigen-specific.

FACTORS AFFECTING ANTIBODY BIOSYNTHESIS

So far, only the direct manifestations of the antibody response have been considered; i.e. the parameters such as antibody concentration, affinity, and heterogeneity which can be used to characterise the response. A study of these parameters alone cannot yield much information about control mechanisms unless we find out how they are influenced by various aspects of the immunisation procedure. For example, the properties of the antigen, its dose and administration, the use of adjuvants and the use of passive antibody are all factors which may influence the characteristics of the antibody response.

The Importance of Antigen Dose

The amount of antigen administered to an individual will have a definite affect upon the subsequent antibody response. Thus, the chicken has been found capable of producing measurable amounts of antibody following immunisation with as little as 40 µg of HSA (Steinberg, Munro, Fleming, French, Stark and White, 1970). The maximum levels of antibody reached following immunisation with larger amounts of antigen, e.g. up to 50 mg of HSA, were progressively but not proportionately greater. It has already been mentioned on page 11 that a characteristic of the antibody response in chickens immunised by a single intravenous injection of antigen was the steady exponential decline in serum antibody levels which occurred from about 7 days after immunisation. The half-life of the antigen-binding capacity during this exponential decay was found to be independent of the peak level of antibody in the serum, and by inference therefore, to bear no relation to the dose of antigen. With repeated injections of large doses of antigen it has proved possible to render chickens tolerant to that antigen (French, Stark and White, 1970).

The effect of antigen dose upon the affinity of antibody produced, and upon the maturation of affinity, has already been referred to on page 15. Previous findings were generally compatible with a theory of cell selection, in that a negative correlation existed between the total number of specific receptors occupied and the average binding affinity of antibodies produced. In other words, high doses of antigen selected for stimulation of a greater total number of cells, including many of low affinity, whilst smaller doses

caused stimulation of fewer cells because those of low affinity were effectively ignored. In chickens, Yamaga and Benedict (1975a) found that repeated immunisation, irrespective of the priming dose, the frequency of injections or the total number of injections, caused biosynthesis of uniformly low amounts of low affinity antibody.

The Mode of Administration of the Antigen

The presentation of antigen is another factor which may govern the eventual features of an antibody response. Variations in the route of injection (e.g. intravenous, intramuscular, intraperitoneal, sub-cutaneous, etc.) do not generally affect the quantity, quality or duration of a response significantly - and the injection site of preference will depend primarily upon the type and amount of antigen, whether adjuvant is included, and the animal being immunised. However, it has been claimed that the route of injection of antigen is an important consideration when attempting to immunise chickens with haptens; for example, Orlans, Saunders and Rose (1968) reported that birds given one injection of DNP-BGG intravenously or subcutaneously with FCA, or multiple injections intramuscularly, responded neither to the hapten nor to the carrier moieties of the antigen. DNP-BGG injected directly into the spleen elicited production of anti-BGG only, whilst two subcutaneous injections of antigen in FCA produced high levels of anti-DNP.

The Use of Adjuvants

The inclusion of antigen in the aqueous phase of a water-in-oil emulsion (with or without mycobacteria) has been shown to stimulate

characteristic patterns of antibody production in chickens (French, Stark and White, 1970). As with a straightforward intramuscular or intravenous injection of antigen in saline, there was an initial burst of antibody biosynthesis starting with the appearance of detectable antibody in the serum about 4 days after immunisation, reaching a peak level between 7 and 12 days, and thereafter falling off at an exponential rate. However, when complete or incomplete adjuvant was used, there was an additional second phase of antibody production; this began approximately 21 days after immunisation and reached maximum levels about 3 weeks later (French, Stark and White, 1970). This biphasic pattern of antibody production was found when antigen was given either in Freund's complete or incomplete adjuvants; in both cases the first phase of antibody production was of similar magnitude to that observed for immunisation with antigen in saline. For incomplete adjuvant the second phase gave comparable antibody levels to those in the initial phase, whereas the use of complete adjuvant provoked a second phase response in which serum antibody levels were usually significantly greater compared to those in the first phase. In addition, it was claimed that the avidity of anti-HSA produced during the second phase of antibody production following immunisation with HSA in either Freund's complete or incomplete adjuvant was greater than the avidity of antibody produced in the first phase (French, Stark and White, 1970). There has been recent evidence that a single intramuscular injection of DNP-BGG in Freund's complete adjuvant caused production of a restricted population of high affinity 7S antibodies (Yamaga and Benedict, 1975b). Generally however, there has been very little comparative work done to explore the effects of adjuvants on

antibody affinity (in fact most experimentation on antibody affinity has been done by routine immunisation of animals with antigen in adjuvant by force of habit), although in mammals too it has been reported that the use of adjuvants was responsible for increased affinity of antibody (Soothill and Steward, 1971; Kim, Greenbaum, Davis, Fink, Werblin and Siskind, 1975); this has been attributed by Mond, Kim and Siskind (1974) to the fact that non-specific augmentation of the immune response (e.g. by injecting FCA or endotoxin with the antigen) favoured more efficient selection of high affinity antibody producing cells.

The Importance of the Carrier in Anti-Hapten Antibody Responses

In the study of anti-hapten antibody responses it has been found empirically that it was essential for the hapten to be coupled to an immunogenic macromolecular carrier before successful immunisation against the hapten could be achieved (Landsteiner and Simms, 1923; Schlossman, Yaron, Ben-Efraim and Sober, 1965). Most commonly used carriers have been serum proteins (globulins and albumins), very large molecules such as haemocyanin, and particulate carriers such as erythrocytes; more exotic carriers have been tried, and these have included polyurethane sponge (Borek and Battisto, 1971) and Brucella organisms (Litman, Chartrand, Finstad and Good, 1973). In guinea pigs, carrier molecules have been graded in terms of their ability to promote production of anti-hapten antibodies of high affinity; i.e. BGG > OVA > BSA > Gelatin > Guinea pig albumin, in terms of carrier effectiveness (Siskind, Paul and Benacerraf, 1966). The actual

density of substituents on the carrier has been shown to affect the amount and quality of antibody (Larralde and Janof, 1972) - possibly as a result of increased receptor affinity for multivalent ligands due to co-operative binding effects. It has already been mentioned on page 30 that the function of a carrier molecule could not be explained solely on the basis of the carrier providing a suitable environment for each haptenic determinant and thereby conferring immunogenicity. The specific role of the carrier in the induction process became evident in the secondary response; an animal immunised for the first time with a hapten-carrier conjugate generally produced an appreciable secondary response to that same hapten only if, for secondary stimulation, the hapten was coupled to the same (homologous) carrier as used in the first instance (Ovary and Benacerraf, 1963; Rajewsky, Schirrmacher, Nase and Jerne, 1968). This phenomenon has been called the "carrier effect," and was still observed for example when the hapten and carrier were separated spatially by interposing a long molecular chain spacer between them (Mitchison, 1967; Ungar-Waron et al., 1973). There has latterly been a considerable amount of work done which supports the concept of a specific co-operative function of carrier determinants in the induction of the response to the hapten. e.g. (1) The induction of tolerance towards the carrier molecule gave rise to an associated depression of the corresponding anti-hapten antibody response upon subsequent challenge with hapten-carrier conjugate (Rajewsky, Rottländer, Peltre and Müller, 1967; Green, Paul and Benacerraf, 1968). (2) Pre-sensitisation with free carrier has been found to enhance the

subsequent anti-hapten response following immunisation with hapten-carrier conjugate (Rajewsky, Schirrmacker, Nase and Jerne, 1968; Katz, Paul, Goidl and Benacerraf, 1970; Paul, Katz, Goidl and Benacerraf, 1970; Hurme, Kontiainen, Seppälä and Mäkelä, 1973; Sarvas, Mäkelä, Toivanen and Toivanen, 1974). This pre-sensitisation presumably served somehow to expand the carrier-specific halper T cell population (Katz, Paul and Benacerraf, 1973). In confirmation of these findings it has been possible to demonstrate that hapten-carrier co-operation was brought about by the interaction of hapten-primed B cells with carrier-specific T cells (Niederhuber, Möller and Mäkelä, 1972). (3) The susceptibility of specific T helper cells to lethally radioactive carrier has been demonstrated (Roelants and Askonas, 1971). (4) Immunisation with hapten coupled to a T-independent carrier (e.g. pneumococcal polysaccharide type III or bacterial levan) elicited a thymus-independent IgM anti-hapten response (Klaus and Humphrey, 1974).

The Effect of Administration of Passive Antibody and its Possible Connection with Antibody-Mediated Negative Feedback Homeostasis

Specific antibody administered before, at the same time as or following active immunisation with the corresponding antigen, has the potential to suppress the production of antibodies by the animal to that antigen. This effect has been demonstrated for a wide range of antigens; e.g. proteins (Uhr and Baumann, 1961; Dixon, Jacot-Guillarmod and McConahey, 1967), flagella (Horibata and Uhr, 1967), bacteriophage (Finkelstein and Uhr, 1964), erythrocytes (Rowley and Fitch, 1964; Möller and Wigzell, 1965; Wigzell, 1966; Axelrad and

Rowley, 1968), tumour antigens (Möller, 1963) and haptens (Brody, Walker and Siskind, 1967). It is reasonable to suppose that the antibody produced during a response may be as effective as passively administered antibody in controlling or limiting the response. It is clear that some mechanism for controlling the antibody response must exist, and Uhr and Möller (1968) have suggested that the mechanism for this may well be antibody-mediated negative feedback. In the elucidation of the mechanisms of antibody-mediated immunosuppression (and by implication, the mechanisms of negative feedback), the importance of the specificity, dosage, timing, antibody class, affinity and the presence of the Fc portion of passive antibody has been comprehensively studied by a great many workers.

The first important question concerns specificity - does antibody specific for a given determinant suppress the response to that determinant alone or does it suppress the response to other determinants on the antigen molecule? In support of the first suggestion, that immunosuppression occurs at the level of individual determinants, it has been shown that to satisfactorily suppress antibody production to key-hole limpet haemocyanin then sufficient passive antibody to cover all the determinant sites was required (Dixon et al., 1967). Further experimental evidence came from the observation by Benacerraf and Gell (1959) that fixation of rabbit anti-BGG onto picrylated-BGG had no effect upon the antigenicity of the hapten; in contrast, immunisation by means of picrylated-BGG or picrylated-OVA with the picryl group blocked by specific antibody did not lead to delayed reactivity to the picryl group. Similarly, passive antibody specific for certain proteolytic fragments of human or guinea pig

globulin did not suppress formation of antibody to all the globulin determinants, but only formation of those antibodies directed against the fragments concerned (Cerottini, McConahey and Dixon, 1969b; Pincus and Nussenzweig, 1969; Pincus, Lamm and Nussenzweig, 1971). In the same way, passive antibody to a certain hapten suppressed antibody production to that hapten only - and not to a second hapten when an animal was immunised with both haptens coupled to the same carrier (Brody, Walker and Siskind, 1967). Likewise, the secondary response to a hapten-protein conjugate was suppressed only in the hapten-specific component by passive anti-hapten antibody (Hamaoka, Takatsu and Kitagawa, 1971). In contrast to these results, there is also a considerable amount of evidence indicating that immunosuppressive antibody acts on the complete antigen rather than on single determinants. Thus, passive antibody against the Forssman antigen was also immunosuppressive for the isophile antigen in animals immunised with SRBC (Greenbury and Moore, 1968). In some cases it was possible to entirely inhibit the response to gammaglobulin molecules with passive antibody directed specifically against certain proteolytic fragments of the globulin (Henney and Ishizaka, 1968). It has also been demonstrated that either carrier-specific or hapten-specific antibodies were separately capable of suppressing the antibody response to both moieties of a hapten-protein conjugate (Henney and Ishizaka, 1970).

The fact that passive antibody appeared to act sometimes on a single determinant yet at other times suppressed the response to whole antigens may reflect the existence of two mechanisms of antibody-

mediated immunosuppression: i.e. (1) A peripheral mechanism whereby passive antibody merely masks the antigenic determinants and deprives the antigen of its immunogenicity (Uhr and Möller, 1968). (2) A central mechanism in which the antibody in the circulation (probably in the form of antigen-antibody complexes) has a suppressive effect on the antibody-producing cells (Uhr and Baumann, 1961; Feldmann and Diener, 1970).

If both postulated mechanisms for antibody-mediated immunosuppression are operational, then the dose of passive antibody could be critical. A small dose of passive antibody would only be expected to act "centrally" (or not at all), being insufficient to have any significant masking effect. On the other hand, a very large dose of passive antibody would only act peripherally - as the immune complexes which it would form would have no free determinants to interact with antibody-producing cells, therefore excluding any central inhibition. It might be anticipated that doses of passive antibody somewhere between these two extremes would exert a dual immunosuppressive force and therefore be the most effective. It has been shown experimentally in fact that the degree of immunosuppression was directly related to the dose of passive antibody (Pierce, 1969), but it has also been pointed out that there appeared to be a definite optimum amount of passive antibody, above and below which immunosuppression was less convincing (Feldmann and Diener, 1970; Diener and Feldmann, 1970). It must not be forgotten that passive antibody (especially IgM and small amounts of high affinity antibody) has, on occasion, augmented an immune response - this has been interpreted as a

specific concentrating mechanism for antigen, for example in the spleen (Pearlman, 1967; Walker and Siskind, 1968; Dennert, 1971).

Passive antibody has been found effective if administered prior to, together with, or after the antigen in suppressing antibody formation to that antigen; in the last instance however there was a definite time lag before any immunosuppressive effect was evident (Möller and Wigzell, 1965; Wigzell, 1966) - the inference being that passive antibody has no inhibitory effect upon cells already committed to synthesising antibody. Moreover, if normal lymphoid cells were incubated with antibody *in vitro* (Möller, 1964) or spleen cells were taken from passively immunised donors (Ryder and Schwartz, 1969), and in each case these cells were transferred into lethally irradiated recipients and challenged with antigen, then a conventional response in terms of antibody production was elicited. This implicated an absolute requirement for both antigen and antibody being present at the same time for immunosuppression to occur.

In correlating the concepts of negative feedback with the proposed switch from IgM to IgG antibody production, it might be expected that IgG antibody specifically inhibits IgM antibody biosynthesis. Experiments have in fact shown that 7S antibody was more efficient *pro rata* in this respect (Finkelstein and Uhr, 1964; Wigzell, 1966) and that 7S antibody specifically depressed 19S antibody production *in vivo* (Sahiar and Schwartz, 1964). In the same way it was found that 19S antibody production was more sensitive to suppression by passive antibody than the 7S response - which in fact became more resistant with time (Wigzell, 1967). It has also been

claimed that in mice immunosuppressive function was subject to sub-class restriction, IgG_1 being more effective than IgG_2 (Murgita and Tokuda, 1967; Murgita and Vas, 1972).

The amount of antibody in the circulation may therefore be related to the course of a natural infection. Initially, when there is little antigen available, the two positive feedback loops (T cell helper function and IgM concentrating mechanism) will be operative. Thereafter, no useful purpose is served by continued and unlimited escalation of antibody production, and at this point negative feedback homeostasis would come into action.

Passive antibody administered at doses large enough to act by the peripheral mechanism would be expected to compete with antigen-binding cells for free antigen, thereby preventing low affinity cells from being stimulated. In this way, any antibody which was produced would be of high affinity; observations of increased antibody affinity under such conditions have been reported (Siskind, Dunn and Walker, 1968). On the other hand, if antibody acts centrally and in the presence of antigen, then a relative decrease in the affinity of any antibody formed might be expected. Such a decrease in antibody affinity has been demonstrated experimentally (Walker and Siskind, 1968). In terms of effectiveness, it has been found that an equivalent amount of high affinity antibody was more inhibitory than low affinity antibody (Walker and Siskind, 1968), and this could be connected with the finding that passive antibody from a late primary response was more immunosuppressive than material from the early phase.

The proposed existence of two ways in which passive antibody might be immunosuppressive could be related to the functional structure of the antibody molecule itself. Straightforward masking of antigenic determinants would require no more than the $F(ab')_2$ antibody fragment whereas inactivation at the cellular level might logically involve both recognition, $F(ab')_2$, and effector, Fc, moieties of the antibody molecule. The apparent effectiveness of intact IgG antibody molecules has been contrasted to the limited immunosuppressive capabilities of $F(ab')_2$ antibody fragments alone (Sinclair, Lees and Elliot, 1968; Sinclair, 1969; Sinclair, Lees, Chan and Khan, 1970; Chan and Sinclair, 1971; Lees and Sinclair, 1973). Furthermore it has been demonstrated that $F(ab')_2$ fragments would compete with, and thereby diminish the immunosuppressive potency of intact IgG antibodies when the two were administered together (Chan and Sinclair, 1973). In chickens, selective removal of cytophilic antibody by adsorption with spleen cells considerably reduced the immunosuppressive qualities of the antiserum thereafter (Iványi, 1970). Conflicting reports stating that the Fc portion of the antibody molecule was not required for immunosuppression have also been published (Tao and Uhr, 1966; McConahey, Cerottini and Dixon, 1968; Cerottini, McConahey and Dixon, 1969a; Chang, Schneck, Brody, Deutsch and Siskind, 1969; Feldmann and Diener, 1972).

To summarise, immunosuppression by passive antibody and probably by circulating antibody would appear to involve at least two processes: (1) The binding of passive (or circulating) antibody to

antigenic determinants, effectively masking these. (2) The inhibition of the antigen-sensitive cells themselves, a central effect as proposed by Uhr and Baumann (1961) and Rowley and Fitch (1964) in which the number of immunocompetent cells capable of responding to antigenic stimulation is reduced. This second mechanism has been developed into a tripartite model by Chan and Sinclair (1971), according to which, the binding of specific antibody to an antigenic determinant can inhibit an antigen-sensitive cell which attaches to a neighbouring determinant on the same antigen: this inhibition is perhaps effected by the Fc portion of the attached antibody molecule. A scheme of this sort is supported by evidence that there are receptors on B lymphocytes specific for the Fc portion of immunoglobulin molecules (Basten, Warner and Mandel, 1972).

It is quite probable that the regulation of antibody biosynthesis in terms of homeostasis is effected by circulating antibody which may act in a similar fashion to antibody administered passively. Control of antibody production by antibody-mediated negative feedback would achieve the inhibition of prolonged and uneconomical antibody biosynthesis. In agreement with this, the removal of specific antibody from the circulation has been shown to cause temporary alleviation of such negative feedback (Graf and Uhr, 1969; Bystryn, Graf and Uhr, 1970).

The scope of this thesis was therefore to investigate certain aspects of the inducibility and specificity of antibody biosynthesis in the chicken. The evidence concerning control mechanisms was obtained from experimental measurements of antibody levels and of the affinity, avidity and heterogeneity of antibody. The influence of a number of factors on these variables, e.g. the effect of antigen, the type, dose and administration upon the subsequent biosynthesis of antibody in chickens was examined; so too were the effects of adjuvants and of passive antibody. The relationship between these factors governing biosynthesis and the characteristics of the antibody response are discussed in terms of a process of clonal selection by antigen followed by cellular differentiation and proliferation.

MATERIALS AND METHODS

A. IMMUNISATION

1. PREPARATION OF MATERIALS FOR INJECTION

Human Serum Albumin

The human serum albumin (HSA) used for experiments was obtained in a purified crystalline state from Behringwerke AG, Marburg Lahn, Germany. For immunisation the required total amount of purified crystalline HSA was dissolved in saline (0.15 M NaCl).

Haptenated Proteins

Solutions of DNP-protein conjugates in PBS (phosphate buffered saline; 0.01 M phosphate buffer pH 7.2, containing 0.15 M NaCl) were stored frozen at -20°. When required a DNP-protein conjugate was allowed to thaw at room temperature, clarified if necessary by centrifugation, and the concentrations of DNP and protein were confirmed by spectrophotometry (as described on page 56) immediately before injection.

Adjuvant Mixtures (water-in-oil emulsions)

(i) Freund's Incomplete Adjuvant (FIA) was prepared from the following reagents: antigen dissolved in saline; Drakeol 6VR, a mineral oil manufactured by The Pennsylvania Refining Company, Butler, Pennsylvania, U.S.A.; Arlacel A, an emulsifying agent manufactured by the Atlas Powder Co., Wilmington, Delaware, U.S.A. Three volumes of Drakeol 6VR were mixed with one volume of Arlacel A in a screw-capped glass vessel (containing one glass bead, diameter 5 mm) and emulsified with

one volume of antigen solution in saline as follows. One third of the antigen solution was taken up in a disposable hypodermic syringe and forcibly ejected into the oil mixture through a 0.5 mm needle. The container was then capped and shaken vigorously before the next portion of antigen solution was added in the same way. After the third and final addition of antigen, the mixed emulsion was taken up into the syringe through a wide bore (1 mm) needle and squirted out again through the fine bore (0.5 mm) needle. The container was then capped and shaken vigorously on a mechanical shaker for at least 30 minutes to ensure that the aqueous phase was finely dispersed throughout the oil phase. The quality of the emulsion was tested by floating a few drops of adjuvant in a beaker of cold tap water; if the adjuvant mixture was a true water-in-oil emulsion then it remained as discrete drops on the surface of the water and showed no tendency to disperse. Adjuvant mixtures were always prepared on the same day as they were to be used.

(ii) Freund's Complete Adjuvant (FCA) was prepared in essentially the same way as the incomplete adjuvant, but with the addition of mycobacteria to the oil phase of the water-in-oil emulsion.

For immunising chickens, Mycobacterium avium Strain D4ER, equivalent to a dose of 5 mg per bird, was included in the oil phase of the adjuvant. For immunising rabbits, the adjuvant contained Mycobacterium tuberculosis human type Strain C equivalent to a dose of 1.5 mg per animal. In both cases the mycobacteria were weighed out into a sterile glass container and finely ground with a glass bead (5 mm diameter) on a mechanical shaker for 10 minutes. The oil

mixture was then added and shaking continued for a further 10 minutes to ensure an even distribution of mycobacteria throughout the oil mixture.

Both mycobacterial strains were obtained in a heat-killed, freeze-dried form from the Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey, England.

2. EXPERIMENTAL ANIMALS AND IMMUNISATION PROCEDURES

Birds: the experimental work to be reported was carried out in the domestic fowl, Gallus domesticus. Both male and female birds were used, ranging in age from 6 to 20 weeks. White Leghorn (Thornber 808) and crossed White Leghorn/Rhode Island Red randomly bred strains were used.

During inoculation the birds were immobilised lying on their sides, either being held by an assistant or restrained by tying their legs together with string and at the same time tying the wings together with the head tucked back between them. Intravenous (iv) injection was by way of the basilic vein which is a tributary of the brachial vein in the wing of the bird. A hypodermic needle suitable for the size of the vein was selected and inserted in the direction of the blood flow. Intramuscular (im) injections were given into the breast muscle.

Rabbits: New Zealand White rabbits, obtained from Kingford Conies, Great Tey, Colchester, England, were used to raise high titre antisera.

For the production of antisera in rabbits, injections were made intramuscularly into both the left and right calf muscles, so that the maximum number of draining lymph nodes could be utilised. Assistance was required to hold the animal during injection, and for this purpose the rabbit was wrapped in a towel with either the left or right leg held free. The primary injections of antigen (approximately 1 mg in total) were given in Freund's complete adjuvant. A second injection of antigen (1 mg) in saline was given intramuscularly 6 weeks later and the animals were bled at 7 and 10 days after the second injection; further booster injections were given at 6-week intervals.

Disposable needles and syringes: Sterile plastic disposable syringes, sizes 1, 2, 5, 10 and 20 ml were used during the course of experimental work. They were obtained from Becton, Dickinson and Co. Ltd., Dun Laoghaire, Co. Dublin, Republic of Ireland. Gillette Scimitar sterile disposable hypodermic needles were also used, the size depending on the size of the animals and the purpose for which they were intended; they were obtained from Gillette Surgical, Isleworth, Middlesex, England.

3. COLLECTION OF BLOOD SAMPLES, SEPARATION AND PRESERVATION OF SERA

Birds: (1) Heart Puncture. The bird was held firmly, by an assistant, on its back on a flat surface. The size of the syringe and needle were governed by the size of the bird and the amount of blood to be taken, usually a 19 gauge needle and a 20 ml syringe were satisfactory. The syringe was held horizontally and the needle

introduced in a posterior direction at the point of the "V" formed by the clavicle and then advanced into the heart.

(2) Brachial venipuncture. The bird was restrained on its side, as described before, and the feathers in the axillary region were plucked. At this point the brachial vein could be seen running from beneath the pectoral muscle to proceed subcutaneously along the ventral surface of the wing, parallel to the humerus. The initial percutaneous puncture was made into the vein in a direction contrary to that of the blood flow, and after the point of the needle was seen to enter the vein, blood was gently withdrawn.

Blood samples were allowed to clot and contract at 37° in a water bath (for between 1 and 4 hours) and then ringed with a flame-sterilised metal spatula to facilitate contraction of the clot. The clotted blood samples were centrifuged at 750 x g for 10 minutes; the serum was removed by Pasteur pipette and stored at -20° in sealed containers.

Rabbits: Blood was taken from the marginal ear vein. The rabbits were restrained by tightly wrapping them in a towel with only their heads protruding. The hair over the vein was carefully shaved and the ear lightly swabbed with xylene to cause vasodilatation. The vein was compressed at the base of the ear, a small 2-3 mm incision was made across the vein and the blood was allowed to drip into clean, sterile, glass Universal containers. Up to 50 ml of blood could be collected from each rabbit in this way. After collection, a cotton swab was pressed over the incision until bleeding from the vein had

stopped; the ear was then carefully washed and cleaned.

Clotting of blood samples was carried out as described before. The sera were stored at -20°.

B. PREPARATION OF ANTIGENS AND HAPTENS

4. PREPARATION OF DNP-PROTEIN CONJUGATES

DNP-protein conjugates were prepared either for the immunisation of chickens or for use in hapten-binding assays; the preparative procedure was adapted from the method of Eisen (1964b).

Protein solutions contained 200 mg of protein; e.g. human serum albumin (HSA), human gamma globulin (HGG; Blood Transfusion Service, Law Hospital, Lanarkshire, Scotland), ovalbumin (OVA; Sigma Chemical Co. Ltd., London, England), chicken serum albumin (CSA; prepared from chicken serum) and haemocyanin (HCN; prepared by differential centrifugation from the blood of Helix pomatia) were used as carrier proteins. These solutions were made up to 10 ml in carbonate/bicarbonate buffer (0.5 M, pH 8.4). To the protein solution (approximately 20 mg ml^{-1}) was added 0.1 ml of a 10% (v/v) solution of 2,4-dinitrofluorobenzene (DNFB; British Drug Houses Ltd., Poole, England) in 1,4-dioxan (British Drug Houses Ltd., Poole, England) and the mixture was allowed to react overnight at room temperature (to obtain less intensely substituted protein molecules the amount of DNFB and/or the reaction time were reduced accordingly).

The hapten-protein conjugate was subsequently separated from uncoupled hapten by gel filtration on a column (30 cm x 1 cm) of

Sephadex G25 (Pharmacia, Uppsala, Sweden) equilibrated with PBS. The DNP-protein conjugate was eluted in the void volume, collected as a single fraction, and stored at -20^{o} without preservatives.

5. DETERMINATION OF THE DNP AND PROTEIN CONCENTRATIONS OF CONJUGATES

Before using DNP-protein conjugates as antigens it was necessary to find the exact concentrations and the molar ratio of hapten and protein moieties. All spectrophotometric measurements were carried out using an SP1800 Ultraviolet Spectrophotometer (Pye Unicam, Cambridge, England) incorporating an SP1806 Synchronised Wavelength Drive Unit and including the facility of a Unicam AR25 Linear Recorder.

(a) DNP concentration: for practical purposes it has to be assumed that all DNP groups attached to the protein are linked by way of the ε-amino group of lysine residues in the protein molecule. It was also necessary to assume that the molar extinction coefficient of conjugated DNP in a protein is the same as that of free ε-DNP-lysine. The DNP molar concentration in a solution of a DNP-protein conjugate can therefore be determined spectrophotometrically - using the information that the wavelength of maximum absorbance (λ_{max}) for DNP-lysine is 360 nm and that the molar extinction coefficient at this wavelength (ε_{360}) is 17,400 mol^{-1} 1. cm^{-1} (Little and Donahue, 1968). In other words, the absorbance (measured through a 1 cm thickness of solution) of DNP-lysine at a concentration of 1 mole $litre^{-1}$ is greatest when measured at 360 nm and at this wavelength has a value of 17,400.

The characteristic peak of absorbance at 360 nm for DNP-protein conjugates, due entirely to the presence of the DNP group, is shown in Figure 3 which is an ultraviolet absorption spectrum of DNP-HSA in solution.

(b) Protein concentration: both the DNP and protein components of the conjugate absorb transmitted ultraviolet light at 280 nm (due to benzene rings in the DNP group and in the aromatic amino acids of the protein). It has already been mentioned that the absorbance of the conjugate at 360 nm is attributable solely to the DNP group and it has been found that the absorbance of ε-DNP-lysine at 360 nm is directly proportional to its absorbance at 280 nm.

i.e. for DNP-lysine $\frac{E_{280}}{E_{360}}$ = constant (c). Various values for this constant have been reported, e.g. 0.361 (Eisen, Kern, Newton and Helmreich, 1959) or 0.385 (Iverson, 1973).

Our own calibration for the absorbance of DNP-lysine at both 280 nm and 360 nm is given in Table 1 and shown in Figure 4. From this calibration, the following values were obtained:

$$\varepsilon_{360} = 16{,}800 \text{ mol}^{-1} \text{ l. cm}^{-1}, \frac{E_{280}}{E_{360}} = 0.321.$$

If E_{280} and E_{360} are the values for the total absorbance of the conjugate at 280 nm and 360 nm respectively, then the value of E_{280} has two components; the contribution of DNP at 280 nm can be calculated as described and is equal to $E_{360} \times c$. Having found this, the contribution from the protein can be calculated and is equal to $E_{280} - (E_{360} \times c)$; i.e. the component of the total absorbance at 280nm attributable to the protein alone = $E_{280} - (E_{360} \times c)$. If the

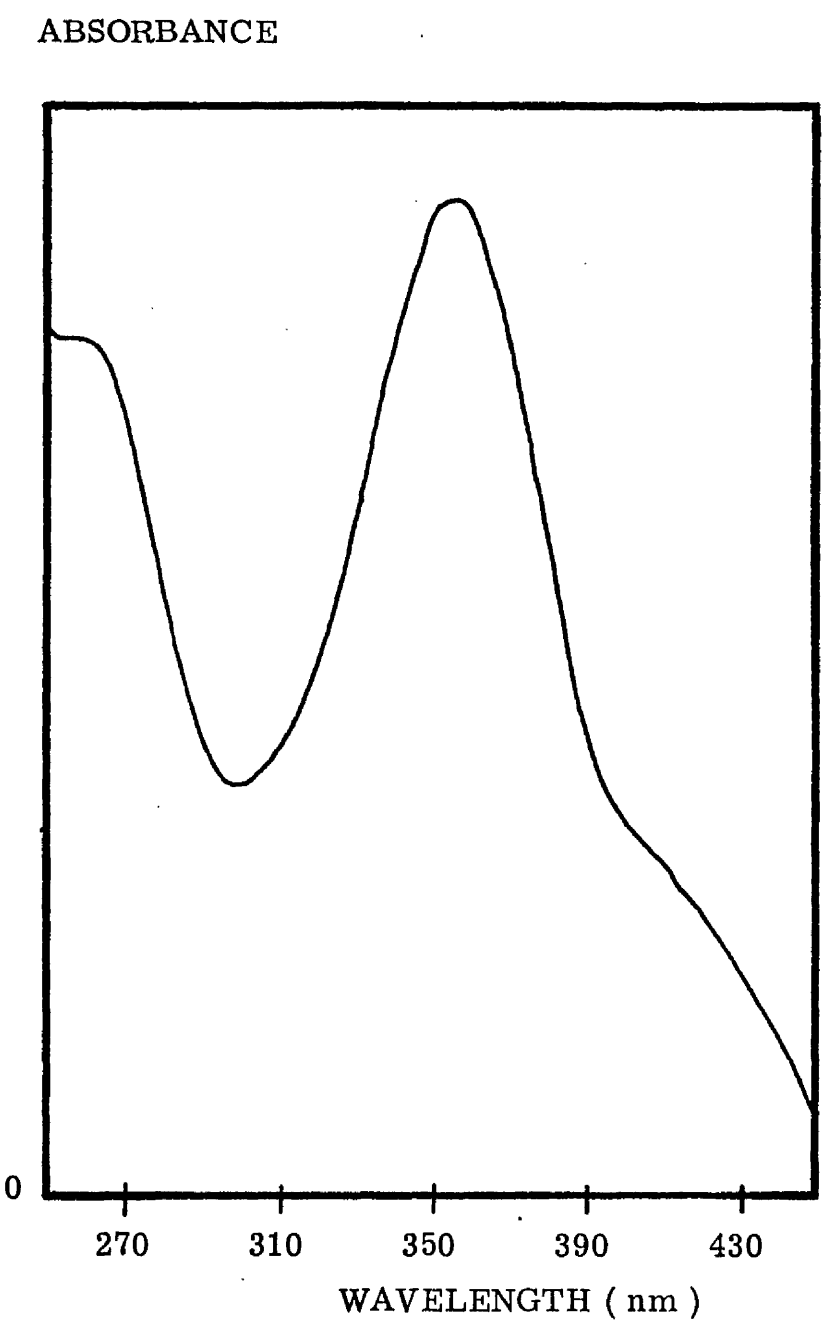


FIG. 3. Ultraviolet absorption spectrum of DNP-HSA.

TABLE 1. Calibration of the Unicam SP1800 Ultraviolet Spectrophotometer by measuring the absorbance of different concentrations of DNP-lysine in a 1 cm cuvette at 280 and 360 nm.

Concentration of DNP-lysine (μmoles litre^{-1})	E_{280}	E_{360}
25	0.116	0.425
50	0.258	0.860
75	0.386	1.250
100	0.546	1.620

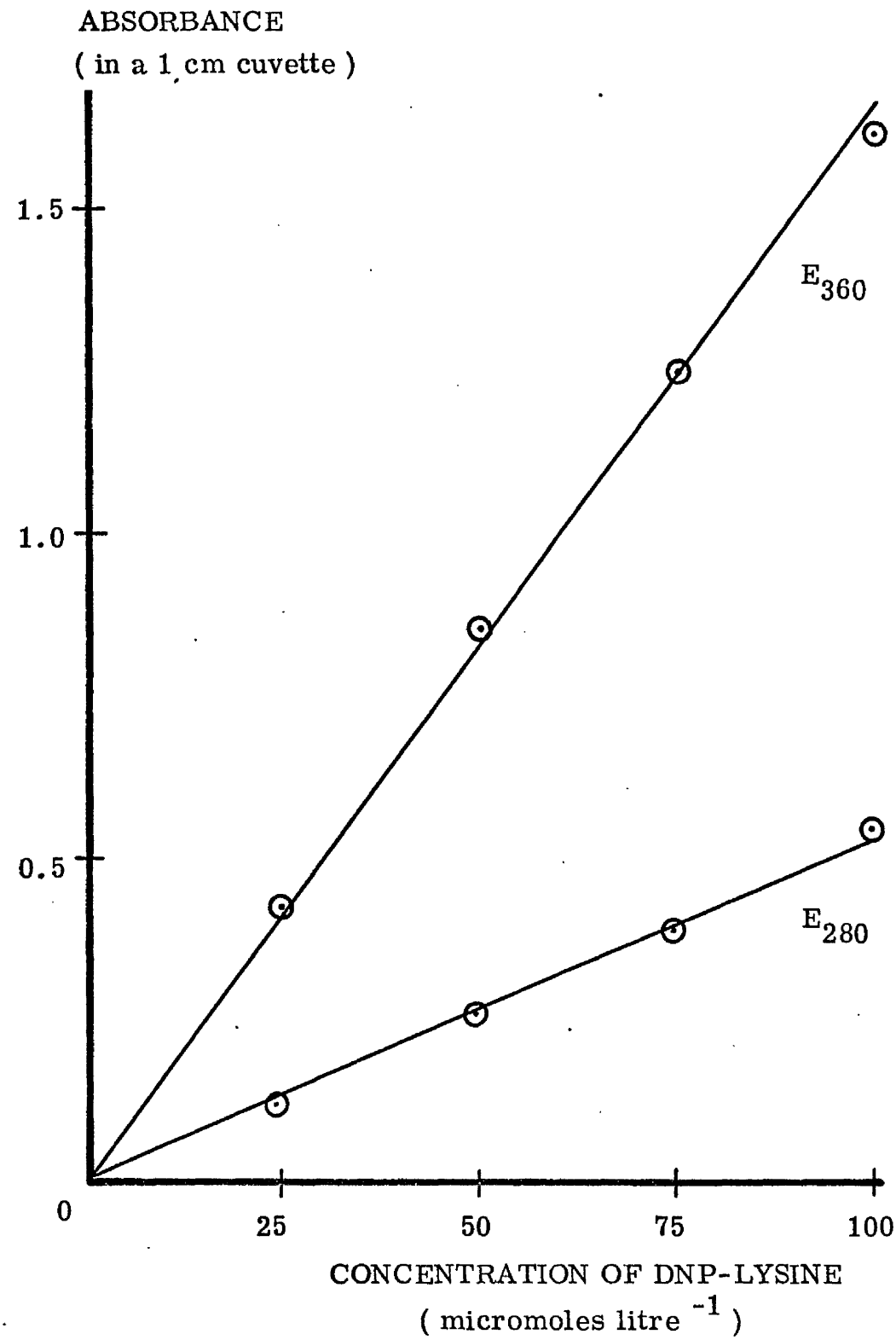


FIG. 4. Calibration graph for the absorbance of DNP-lysine at 280 and 360 nanometres.

protein in question has a molar extinction coefficient = ε_{280} (protein), then the molar concentration of protein in the conjugate =

$$\frac{E_{280} - (E_{360} \times c)}{\varepsilon_{280}\ (\text{protein})} .$$

(c) DNP to Protein Molar Ratio: this was calculated from the following equation, itself derived from the formulae describing the concentrations of DNP and protein in a conjugate.

$$\text{DNP:protein (molar ratio)} = \frac{E_{360}}{\varepsilon_{360}\ (\text{DNP-lysine})} \times \frac{\varepsilon_{280}\ (\text{protein})}{E_{280} - (E_{360} \times c)} .$$

6. PREPARATION OF DINITROPHENYLATED ERYTHROCYTES

To assess the suitability of a particulate entity as a carrier for the DNP hapten, chickens were immunised with heterologous red blood cells to which the hapten had been conjugated. The method for coupling DNP to human erythrocytes was adapted from that recommended by Levine and Levytska (1967).

Human red blood cells were separated from heparinised venous blood by the addition of a $\frac{1}{4}$ volume of 6% dextran in 0.9% sodium chloride (Dextraven 150; Fisons Ltd., Loughborough, England) and allowing the erythrocytes to sediment. The supernatant plasma was aspirated and the red blood cells remaining were washed three times with the coupling buffer (0.05 M carbonate/bicarbonate, 0.15 M NaCl and 0.01 M EDTA, pH 8.0).

A 2 ml volume of packed human red blood cells was added to 13 ml of coupling buffer containing 50 µl of a 10% (v/v) solution of DNFB in acetone. The cells were agitated gently for 1 hour at room

temperature. Thereafter the erythrocytes were washed 3 times in PBS and resuspended in a final volume of 10 ml. This procedure yielded suspensions containing approximately 2×10^9 DNP-HuRBC ml^{-1}.

7. PREPARATION OF DINITROPHENYLATED CELLULOSE

For investigating the effect of immunising chickens with the DNP hapten immobilised on a polysaccharide carrier, a batch of dinitrophenylated cellulose was prepared according to the method recommended by McKay (personal communication).

A 500 mg amount of chromatographic cellulose (DE 22; Whatman Biochemicals Ltd., Maidstone, Kent, England) was suspended in 10 ml of absolute ethanol. To this suspension was added 0.25 ml of undiluted DNFB. The coupling reaction was allowed to proceed overnight at room temperature. The product was then extensively washed consecutively in ethanol, water, dilute alkali and dilute acid until no further colour (indicating free dinitrophenol) appeared in the supernatant fluid.

8. PREPARATION OF DINITROPHENYL HAPTENS

(a) DNP-ε-aminocaproic acid (DNP-EACA).

DNP-EACA was used as the ligand in equilibrium dialysis studies of anti-DNP antibody affinity (and for this purpose was supplemented with small amounts of tritium-labelled DNP-EACA). Because chickens were usually immunised with DNP attached to a protein carrier and since for such conjugates the majority of DNP groups are assumed to be linked to the ε-amino groups of lysine residues, DNP-EACA was selected as a suitable ligand because of its structural similarity to

the dinitrophenyl determinant of DNP-protein conjugates (as compared in Figure 5).

The method used for preparing DNP-EACA was similar to that employed by Werblin and Siskind (1972a) and was carried out as follows.

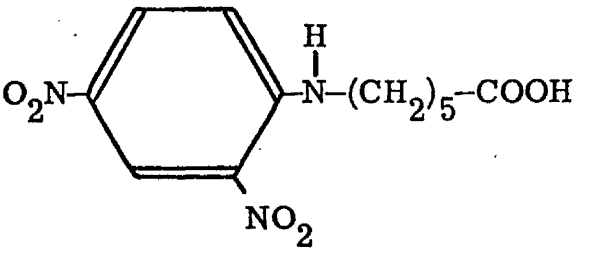
A solution containing 3 g of 6-aminocaproic acid (British Drug Houses Ltd., Poole, England) and 5 g of sodium carbonate was made up to 100 ml with distilled water; 3 ml of a 10% solution (v/v) of DNFB in 1,4-dioxan were added to the solution, which was then stirred for 1-2 hours at room temperature. The product was precipitated with 2 M hydrochloric acid, sedimented by centrifugation and washed with 2 M hydrochloric acid. The DNP-EACA was then washed and finally recrystallised from 50% ethanol.

The dinitrophenyl group has a characteristic peak of absorbance (λ_{max}) at approximately 360 nm. This peak of absorbance is shown in Figure 6, which is an ultraviolet absorption spectrum of DNP-EACA in distilled water; for this solution, λ_{max} = 365 nm.

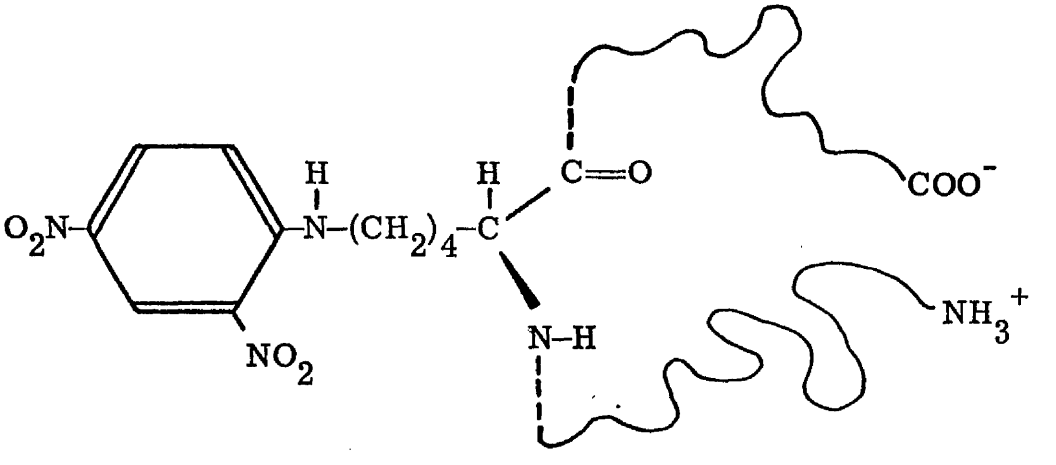
(b) Tritiated DNP-ε-aminocaproic acid, [^{3}H]-DNP-EACA.

[^{3}H]-DNP-EACA was used as the radio-labelled ligand in an equilibrium dialysis system for assessing anti-DNP antibody affinity. The preparation was adapted from the methods employed by Katz, Paul, Goidl and Benacerraf (1970) and by Werblin and Siskind (1972a).

The radio-labelled ligand was prepared as a stock solution in the following way: 2,4-dinitro-1-fluoro[3,5-^{3}H]benzene ([^{3}H]DNFB) was obtained from the Radiochemical Centre, Amersham, England, and was



Representation of the DNP-EACA molecule.



Representation of the DNP group attached to a protein molecule at the ε-amino group of lysine.

FIG. 5. Comparison of the molecular structure of the ligand DNP-EACA with that of the DNP group when it is attached to the ε-amino group of lysine in a protein.

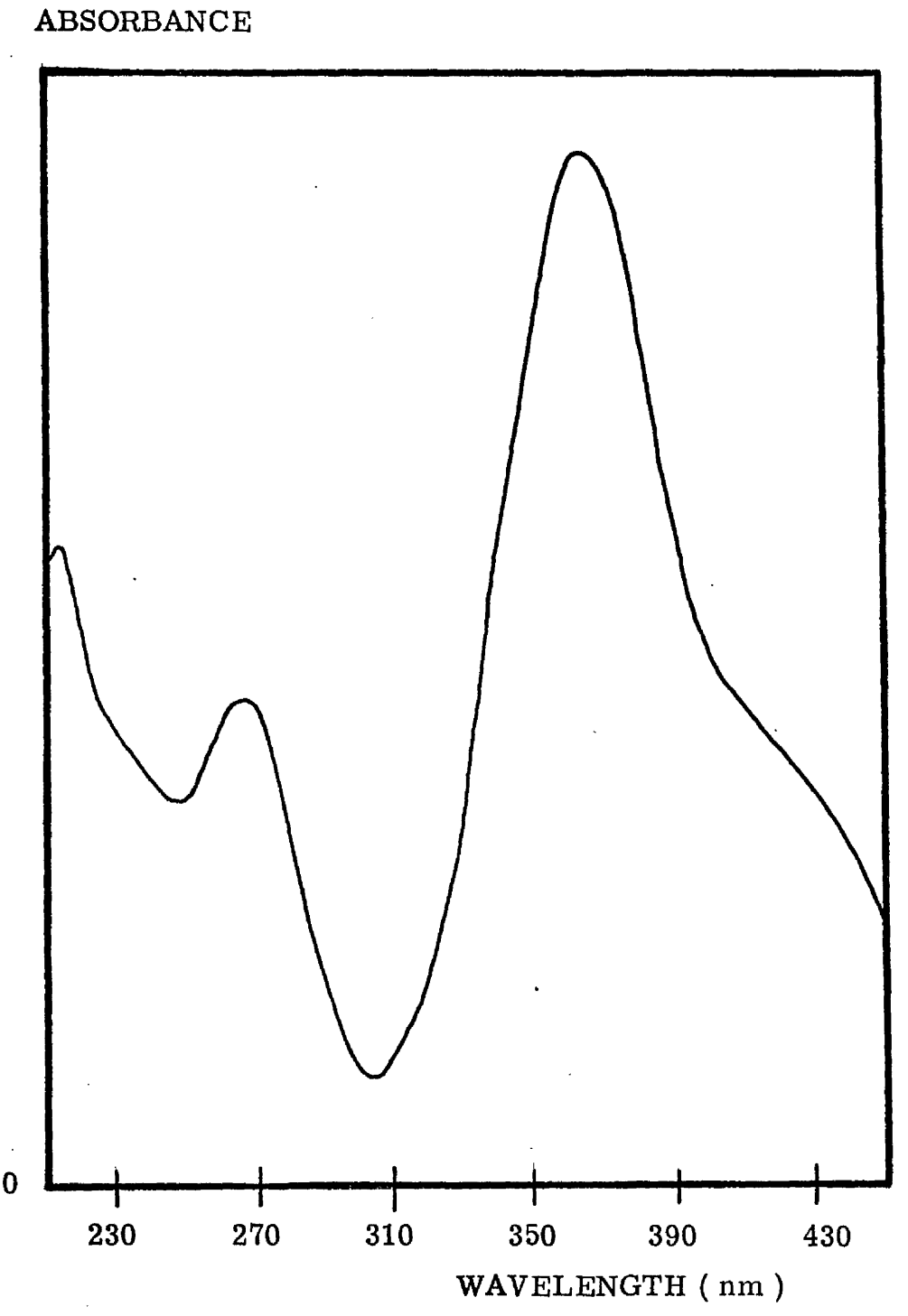


FIG. 6. Ultraviolet absorption spectrum of DNP-ε-aminocaproic acid (DNP-EACA).

supplied as a 1 ml solution in benzene. The radioactivity was 1 mCi ml^{-1} and the specific activity was 13 mCi μmol^{-1}; i.e. 1 ml contained 1 mCi of radioactivity, which was equivalent to 0.077 μmoles of $[^3H]$-DNFB.

A 1 ml volume of $[^3H]$-DNFB was taken to dryness by blowing a steady stream of dry nitrogen over its surface; 0.1 ml of a solution of 6-aminocaproic acid (MW 131.18) in 5% Na_2CO_3, representing a 100-fold molar excess (i.e. 7.7 μmoles or 1.012 mg in 0.1 ml, or 10.12 mg ml^{-1}) was added to the dry $[^3H]$-DNFB and the mixture stirred for 2 hours. The reaction mixture was then applied to a thin layer silica gel chromatographic plate. Chromatograms were developed in n-butanol and the position of the $[^3H]$-DNP-EACA spot was located by correlation with a visible amount of non-radioactive DNP-EACA chromatographed simultaneously. The area of the gel containing the $[^3H]$-DNP-EACA was scraped from the glass plate, and the product was eluted from the scrapings of silica gel by thorough mixing with PBS.

The concentration of $[^3H]$-DNP-EACA was determined as follows: for DNP-EACA λ_{max} = 365 nm and ε_{365} = 17,800 mol^{-1} l. cm^{-1} (Little and Donahue, 1968). For the $[^3H]$-DNP-EACA, prepared as described, E_{365} = 0.99. Therefore, the concentration of $[^3H]$-DNP-EACA = 5.56×10^{-5} M, or 0.0556 μmoles ml^{-1}. By diluting this 1:55.6, a stock solution of $[^3H]$-DNP-EACA at a concentration of 1 nanomole ml^{-1} was obtained.

(c) Divalent DNP hapten. The divalent DNP hapten, 1,6-bis-(2,4-dinitrophenylamino)hexane, i.e. DNP-NH-$(CH_2)_6$-NH-DNP, was used in experimental studies into the feedback inhibition of the anti-DNP

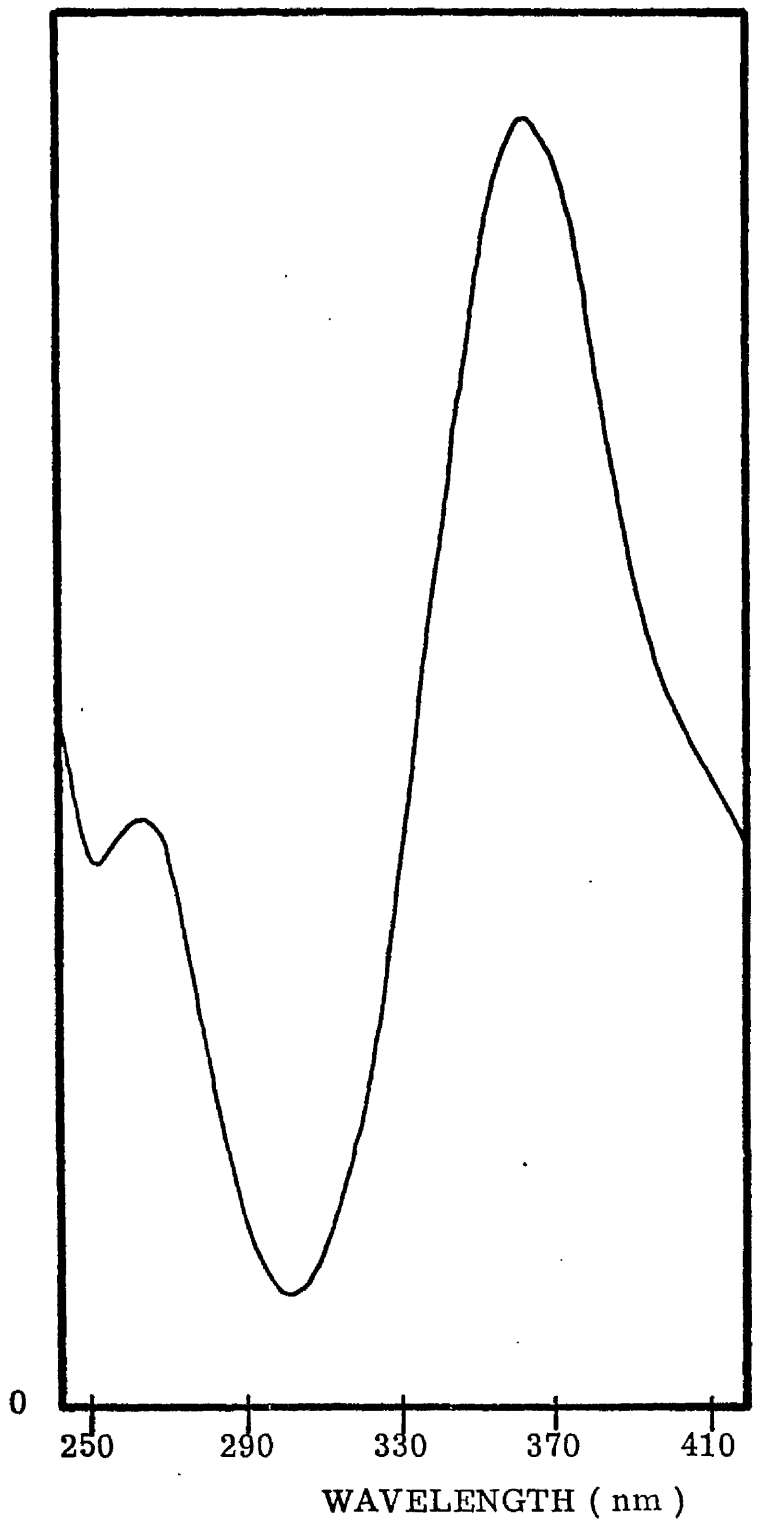


FIG. 7. Ultraviolet absorption spectrum of 1,6-bis-(2,4-dinitrophenylamino)hexane.

antibody response in chickens. The preparation, based on the method described by Green, Wilder and Schumaker (1972), was as follows: 60 millimoles (7.25 ml) of DNFB were made up to 120 ml with absolute ethanol. A solution containing 30 millimoles (3.48 g) of 1,6-diaminohexane (British Drug Houses Ltd., Poole, England) dissolved in 50 ml of 1 M $NaHCO_3$ was also prepared. The two solutions were then mixed and stirred for 2 hours at room temperature; the product precipitated spontaneously. The divalent hapten was washed alternately with ethanol and water three times and then dried.

An ultraviolet absorption spectrum of 1,6-bis-(2,4-dinitrophenylamino)hexane is shown in Figure 7; for the divalent hapten λ_{max} = 362 nm.

9. PREPARATION OF CHICKEN SERUM ALBUMIN (CSA)

Chicken serum albumin was required as a carrier for DNP in the Farr test to measure anti-DNP antibodies (as described on page 80). The method for preparing chicken serum albumin was as follows: a 200 ml volume of methanol containing 0.05 M trichloroacetic acid (TCA) was added to 20 ml of chicken serum. The resulting precipitate was deposited by centrifugation and discarded. Sodium hydroxide (2 M) was added dropwise to the supernatant until the pH (originally about pH 2) had reached 6.0. At this point a white flocculent precipitate was formed. This precipitate was sedimented by centrifugation and the supernatant fluid was discarded; the precipitate was then washed twice with methanol, twice with ether and then air-dried at room temperature.

10. PREPARATION OF RADIO-LABELLED PROTEINS

(a) Radio-iodination of HSA.

HSA was trace-labelled with radioactive iodine (using either ^{131}I or ^{125}I which were supplied as iodide, carrier free, in NaOH solution by the Radiochemical Centre, Amersham, England and were dispensed in millicurie amounts by the Isotope Dispensary, Institute of Radiotherapeutics, Western Infirmary, Glasgow) for use in the measurement of the antigen-binding capacity and the determination of the avidity of chicken antisera. The radio-labelling was carried out using the direct oxidation procedure of Hunter and Greenwood (1962).

In aqueous solution chloramine T is a mild oxidising agent; on addition to a solution of iodide and protein there is a rapid incorporation of iodine atoms into the protein molecules - almost exclusively by formation of mono-iodotyrosine.

The reactants were injected through the rubber seal on a phial containing the thiosulphate-free, radioactive sodium iodide. The exact sequence of these additions, and the concentrations of reagents, are given in Figure 8 which is a "flow" diagram of the labelling procedure.

After sufficient time had been allowed for iodination to take place (3 minutes), sodium metabisulphite was added in slight excess to reduce the chloramine T and any excess iodine; carrier iodide was then added and the reaction mixture was loaded onto a 30 cm x 1 cm column of Sephadex G25. The labelled protein was then

(a) ^{131}I or ^{125}I as carrier-free iodide (e.g. $Na^{131}I$), 1 mCi per mg of protein.

(b) 0.25 ml chloramine T (1 mg ml^{-1} in PBS).

(c) 0.10 ml HSA (10 mg ml^{-1} in PBS).

Shake and allow to react for 3 minutes

(d) 0.05 ml sodium metabisulphite (2.4 mg ml^{-1} in PBS).

(e) 0.20 ml potassium iodide (10 mg ml^{-1} in PBS).

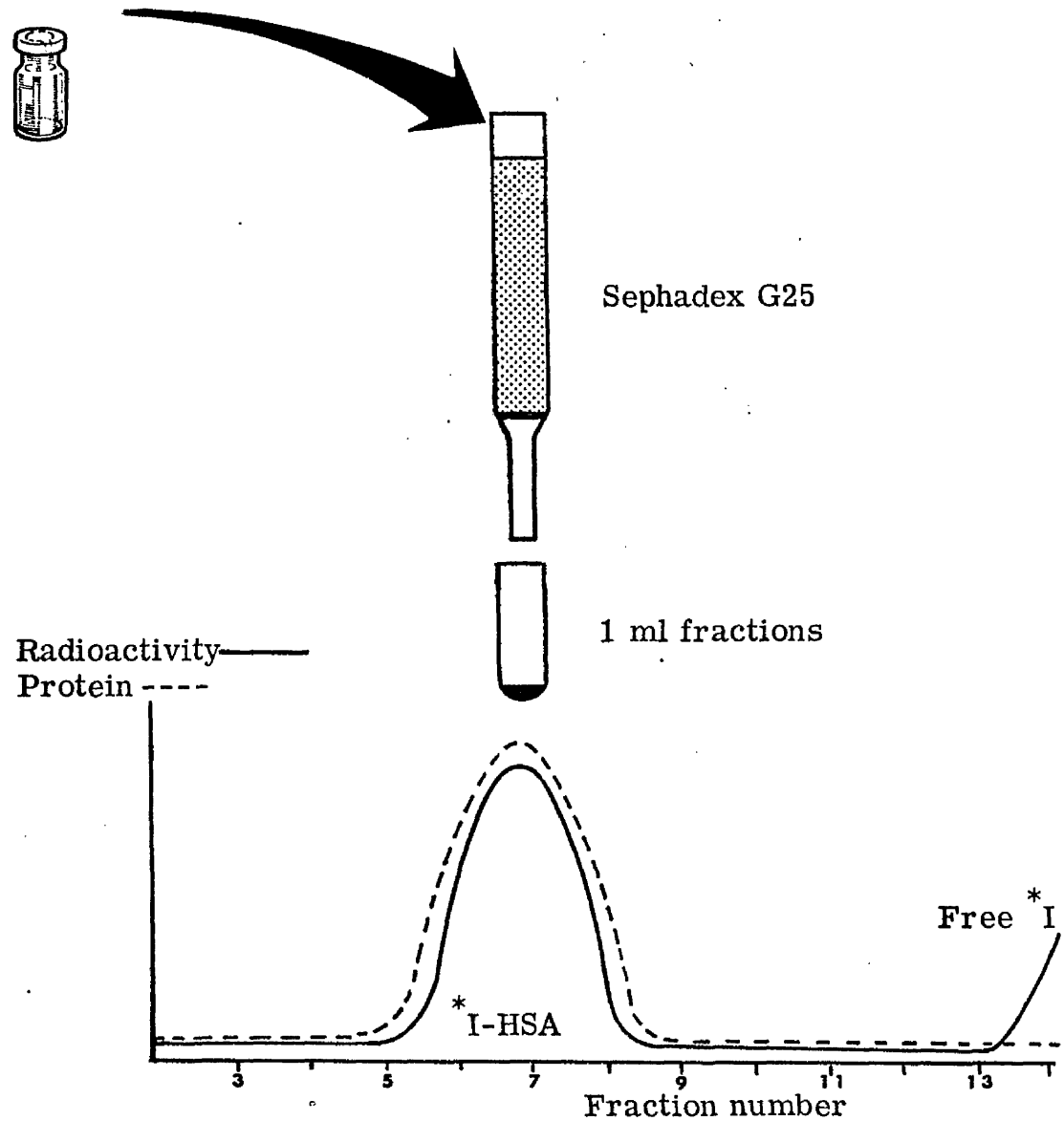


FIG. 8. Procedures for radio-iodinating HSA and for separation of the labelled protein.

separated from unattached radio-iodine by gel filtration; 1 ml fractions were eluted from the column, and the tubes containing the most radioactivity associated with radio-labelled protein were pooled together into a single aliquot. The radioactivity of this 3 ml aliquot was measured on a gamma counter and the HSA concentration was calculated from the spectrophotometric absorbance at 280 nm.

Under these conditions a radio-iodinated HSA solution of 200 to 300 μg ml^{-1} and with a specific activity of 0.85 to 0.90 mCi mg^{-1} was usually obtained.

(b) Radio-iodination of DNP-Proteins.

DNP-proteins were trace-labelled with radioactive iodine for use in the measurement of the hapten-binding capacity of chicken anti-DNP antisera. The method was essentially the same as that used for radio-labelling HSA.

From experience it was found necessary to use a radio-labelled antigen with a low degree of DNP substitution, since heavily dinitrophenylated proteins were partially precipitable in 40% saturated ammonium sulphate. Both ovalbumin and chicken serum albumin were tried as carrier proteins in this system; however, CSA was used in preference to OVA because of the difficulty in attaching adequate amounts of radio-iodine to the inaccessible tyrosine residues of the latter.

C. PREPARATION AND SEPARATION OF IMMUNOGLOBULINS

11. PREPARATION OF IMMUNOGLOBULINS FROM CHICKEN SERUM BY SALT FRACTIONATION

For experiments involving the passive immunisation of chickens

with antibody, an antibody-containing globulin fraction was prepared. Because of the high lipid content of chicken serum there is a tendency for IgM and IgG to aggregate during salt precipitation and for this reason a procedure using sodium sulphate precipitation, comparable to that described by Orlans, Rose and Marrack (1961), was preferred to precipitation with 40% saturated ammonium sulphate. The details of the method are as follows: to a known quantity of chicken serum was added an equal volume of warm (30°) sodium sulphate at a concentration of 0.36 g ml^{-1} (36%) - such that the resulting solution contained 0.18 g ml^{-1} (18%) of sodium sulphate. The solution was allowed to stand at room temperature for 30 minutes, after which the precipitate which had formed was sedimented by centrifugation (M.S.E. Superspeed 50 TC) for 10 minutes at 30,000 x g. The supernatant fluid was removed and discarded, and the precipitate was dissolved to one half of the original serum volume in phosphate buffer (0.1 M, pH 8.0). The globulins were precipitated twice more - each time by the slow addition of sodium sulphate to a final concentration of 0.14 g ml^{-1} (14%) and then centrifuged as before. Following the third and final precipitation and centrifugation, the globulin fraction was dissolved in PBS and dialysed against PBS for 48 hours at 4°. After dialysis, the protein solution was cleared by centrifugation at 2,000 x g for 15 minutes and the concentration of protein determined by measuring the spectrophotometric absorbance at 280 nm.

12. SEPARATION OF 7S AND 19S CHICKEN ANTIBODIES BY ULTRACENTRIFUGATION

For investigating the relative proportions of specific

antibody belonging to either the 7S or 19S class of immunoglobulin (i.e. the isotypic heterogeneity of antibodies) in an antiserum, a method for the complete separation of these two classes was required. The separation was achieved by sucrose density gradient centrifugation using a procedure similar to that recommended by Stanworth and Turner (1973).

Sucrose density gradients were prepared by layering 1 ml volumes respectively of 40%, 30%, 20% and 10% solutions of sucrose in saline (w/v) on top of each other in ½" x 2" plastic centrifuge tubes (Beckman - R.I.I.C. Ltd., Eastfield Industrial Estate, Glenrothes, Scotland). The gradients were equilibrated by leaving overnight at 4°. Antiserum (a 1 ml volume of antiserum diluted 1:50) was carefully layered on top of the sucrose gradient, making a total volume of 5 ml in each tube. Separations were carried out in groups of three by centrifuging the samples in a swing-out SW65 rotor on a Beckman L2-65 preparative ultracentrifuge at 40,000 r.p.m. (114,000 x g at R_{av} = 6.4 cm) for 18 hours at 4°. Following centrifugation the tubes were punctured and 0.25 ml fractions were collected dropwise from the bottom of the tube, under positive pressure, using an automatic fraction collector and drop counter (Beaumaris Instrument Co. Ltd., Beaumaris, Angelsey, Wales).

Once the antibodies in the starting material had been separated according to sedimentation rate, specific antibody residing in each of the two immunoglobulin classes (7S and 19S) was quantitated by measuring the amount of radio-labelled antigen (e.g. ^{131}I-HSA) which was bound by each 0.25 ml fraction. A 0.1 ml sample was taken from

each separated fraction and was thoroughly mixed with 0.1 ml 10% normal chicken serum in PBS and with 0.1 ml radio-labelled antigen (e.g. ^{131}I-HSA at 2 μg ml^{-1}). The tubes were left to equilibrate for 2 hours at room temperature, and then 0.2 ml of saturated ammonium sulphate* was added to each. The precipitates were deposited by centrifugation at 850 x g for 30 minutes, each washed with 0.5 ml 40% saturated ammonium sulphate and centrifuged as before. The final precipitate in each tube was suspended in 0.5 ml PBS and its radioactivity was measured using an automatic gamma counter. (For measuring radioactivity in a large number of separate samples, an ICN Tracerlab Gamma Set 500, which had a maximum load of 500 samples, was used).

Standard controls were obtained by mixing 0.1 ml 10% normal chicken serum, 0.1 ml PBS and 0.1 ml ^{131}I-HSA in a tube with either: (1) for the positive control, 0.3 ml of 10% trichloroacetic acid (TCA) was added. This precipitated all the protein-bound radioactivity and therefore indicated the radioactive counts which would be obtained if 100% of the added antigen was bound by antibody (i.e. a possible maximum of 200 ng of ^{131}I-HSA) in a 0.1 ml sample; or (2) for the negative control, 0.2 ml of saturated ammonium sulphate was added. This selectively precipitated the globulins present but

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*Saturated ammonium sulphate solution: this was prepared by dissolving 550 g of ammonium sulphate (anhydrous) in 600 ml of distilled water. After filtration this solution was made up to 990 ml and the pH was adjusted to 6.5 by addition of concentrated aqueous ammonia. The final volume was made up to 1000 ml with distilled water.

TABLE 2. Antigen-binding, spectrophotometric absorbance and immunoelectrophoresis of fractions, from either chicken anti-HSA antiserum or from normal chicken serum, separated by sucrose density gradient centrifugation (1 ml diluted serum centrifuged for 18 hours at 40,000 rpm and 4°).

Information from the fractionation of specific anti-HSA antiserum, diluted 1:50				Information from the fractionation of normal chicken serum, diluted 1:10	
FRACTION NUMBER	^{131}I-HSA BOUND (cpm)	ng HSA BOUND PER FRACTION	E_{280}	IMMUNOELECTROPHORESIS	
				IgG	IgM
Bottom of tube					
1	25,308	175.6	0.072	-	±
2	7,422	33.3	0.057	-	-
3	5,248	16.1	0.051	-	-
4	4,703	11.7	0.082	±	-
5	16,602	106.4	0.132	±	-
6	27,561	193.5	0.215	++	-
7	40,791	298.8	0.317	+++	-
8	34,425	248.1	0.374	++	-
9	10,453	57.5	0.427	+	-
10	9,912	53.1	0.549	±	-
11	7,984	37.8	0.541	-	-
12	8,124	38.9	0.345	-	-
13	9,409	49.1	0.159	-	-
14	10,162	55.1	0.110	-	-
15	10,944	61.4	0.118	-	-
16	10,364	56.7	0.090	-	-
17	5,279	16.3	0.053	-	-
18	5,556	18.5	0.052	-	-
19	4,521	10.3	0.044	-	-
20	4,169	7.9	0.050	-	-
Meniscus					

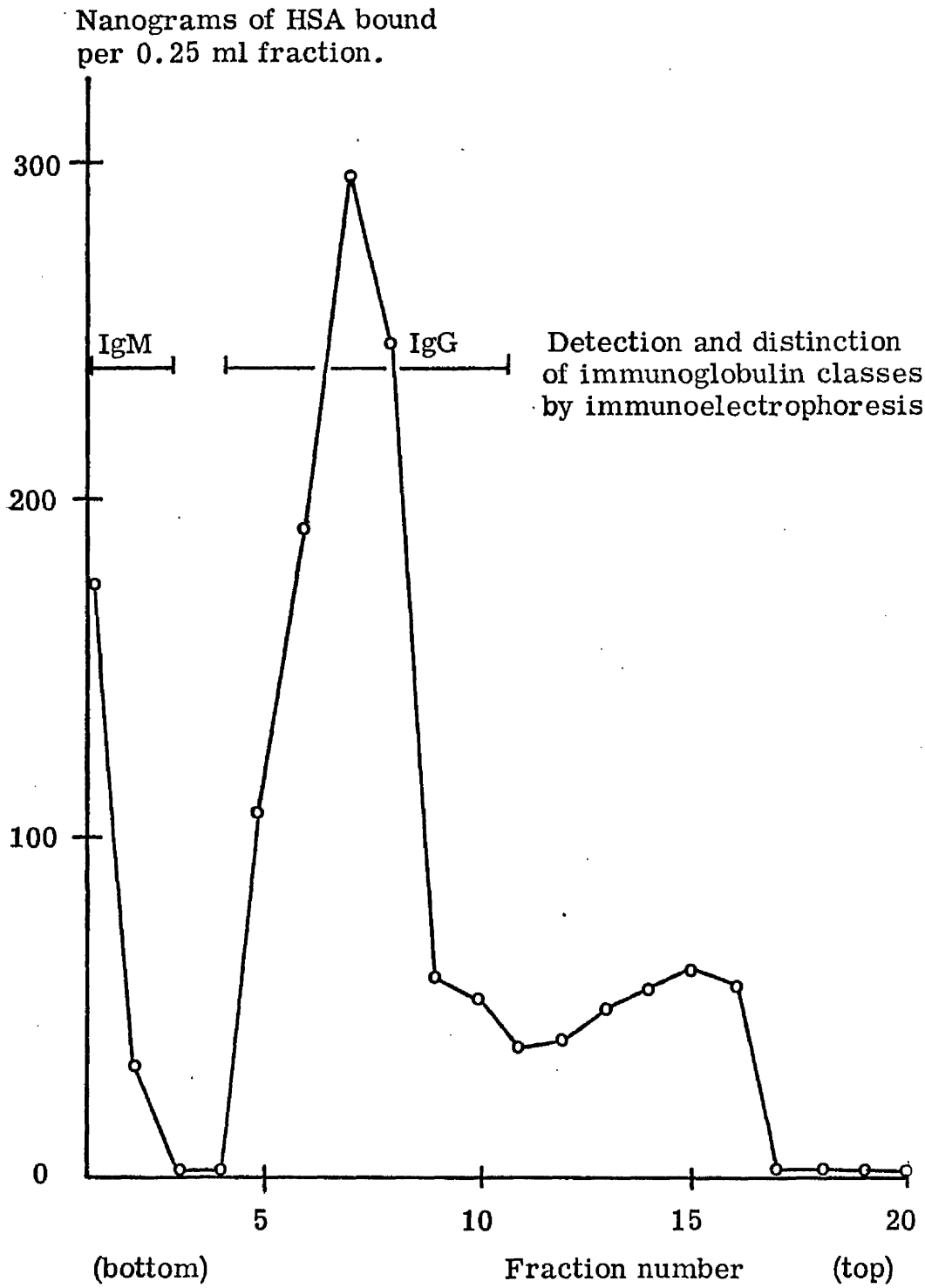


FIG. 9. Distribution of antigen-binding capacity in antiserum fractions separated by sucrose density gradient centrifugation.

not the ^{131}I-HSA and therefore indicated the radioactive counts which would be obtained if none of the added antigen was bound specifically by antibody.

The radioactive count from each tube containing test samples was corrected for non-specific binding of antigen, precipitation of denatured antigen, and for background radiation by subtracting the mean value for the ammonium sulphate control tubes; this corrected value was then expressed as a percentage of the mean value for the TCA control tubes (similarly corrected). This percentage was taken as representing the percentage of the antigen added to each tube which was bound by antibody, and from it was calculated the amount of antigen bound by each 0.25 ml fraction of serum.

The class of immunoglobulin in each fraction was identified by correlation with the immunoelectrophoretic distribution of immunoglobulin classes obtained from a separate fractionation of normal chicken serum (diluted 1/10 for this purpose) as given in Table 2.

Details of a typical separation of chicken anti-HSA antiserum into 7S and 19S antibodies and of the quantitation of each are given in Table 2 and shown graphically in Figure 9. From Table 2, antigen-binding activity in fractions 1, 2 and 3 was attributed to IgM; antigen-binding in all subsequent fractions was attributed to 7S immunoglobulin.

13. EXTRACTION OF IMMUNOGLOBULIN FROM ADJUVANT GRANULOMA TISSUE

An immunoglobulin-containing extract was prepared from

adjuvant granuloma tissue for the purpose of comparing the level of antibody in the tissue at the site of injection with the level of circulating antibody in the serum of chickens. Granuloma extract was also used for experimental passive immunisation of chickens. The extract was prepared as follows:

Chickens which had been immunised 7 weeks previously by a single intramuscular injection of antigen (5 mg HSA) in Freund's complete adjuvant were killed by an overdose of Nembutal (sodium pentobarbitone, 60 mg ml^{-1}) - 50 mg per kg body weight injected intravenously. As much as possible of the adjuvant granuloma which had developed at the site of injection in the breast muscle was excised and the tissue was weighed. Each tissue specimen was then cut into small pieces and immersed in 50 ml saline before being homogenised (M.S.E. homogeniser) for 15 minutes. Fibrous remnants were removed from the homogenates which were then centrifuged at 5000 x g for 15 minutes to sediment debris. The supernatants were decanted and the total volume of each was made up to 50 ml with saline. To each 50 ml extract was added 34 ml of saturated ammonium sulphate (to give a solution 40.5% saturated in ammonium sulphate); the solutions were stirred mechanically for 30 minutes at room temperature. The precipitated globulins were sedimented by centrifugation (750 x g for 30 minutes) and washed twice with 25 ml of 40% saturated ammonium sulphate each time. After the final precipitation and centrifugation the precipitates were dissolved in 30 ml PBS and dialysed against PBS for 48 hours at 4^{o}; the extracts were then finally cleared of any precipitated material by centrifugation.

The antigen-binding capacity of each soluble extract of granuloma tissue was measured by the Farr test. Results were expressed as the ABC_{30} per 100 mg of tissue.

D. DETECTION AND MEASUREMENT OF ANTIBODY

14. DETERMINATION OF THE ANTIGEN-BINDING CAPACITY OF CHICKEN ANTIBODIES

(a) Anti-HSA: the level of anti-HSA antibodies in the serum of immune birds was determined by an ammonium sulphate precipitation method which provided a value for the antigen-binding capacity of the antiserum. The procedure was a modified version of the technique described by Farr (1958) and was carried out as follows.

The antigen used in the system was HSA, trace-labelled with either ^{131}I or ^{125}I, and used at a concentration of 1 $\mu g\ ml^{-1}$ and at a specific activity of 0.85 to 0.90 mCi per mg HSA.

One tube contained undiluted test serum and a further seven tubes (soda glass, 2" x $\frac{3}{8}$") contained the test serum diluted serially X 2 (i.e. each antiserum was diluted from neat in the first tube, to 1/128 in the eighth and final tube in the series) in 10% normal chicken serum (in PBS). The final volume in each tube was 0.1 ml. Radio-labelled HSA (0.2 ml at 1 $\mu g\ ml^{-1}$) was added to each tube and the contents were mixed thoroughly (vortex mixer; A. Gallenkamp and Co. Ltd., Stockton-on-Tees, England). All the tubes were then left overnight at 4° to allow complete equilibration. On the next day, 0.2 ml of saturated ammonium sulphate was added to each tube - this caused precipitation of the globulin fraction, including any radio-

labelled antigen bound to antibody; antigen not attached to antibody remained in solution. Precipitates were deposited by centrifugation at 850 x g (for 30 minutes) and the supernatants were carefully removed; the precipitates were then washed by addition and mixing with 0.5 ml of 40% saturated ammonium sulphate and centrifuged as before. After removal of supernatant fluid from the final precipitates, each was suspended in 0.5 ml PBS; the radioactivity in each tube was then measured using an automatic gamma counter.

Standard controls were obtained, in duplicate, by mixing 0.1 ml 10% normal chicken serum with 0.2 ml radio-iodinated HSA (1 μg ml^{-1}) in a tube and then adding either: (1) for the positive control, 0.3 ml of 10% TCA was added. This precipitated all the protein-bound radioactivity and therefore indicated the radioactive counts which would be obtained if 100% of the added antigen was bound by antibody; or, (2) for the negative control, 0.2 ml of saturated ammonium sulphate was added. This selectively precipitated the globulins present but not the *I-HSA and therefore indicated the radioactive counts which would be obtained if none of the added antigen was specifically bound by antibody, and at the same time allowed for non-specific binding, precipitation of denatured antigen and background radioactivity to be corrected for in the test samples.

(b) Anti-DNP: the quantitation of anti-DNP antibodies in the serum of immune birds was effected in exactly the same way as the measurement of anti-HSA antibodies described in the previous section, by using a modified Farr test. However, in this case the antigen used was

radio-iodinated DNP-CSA (DNP-*I-CSA); for each test it was used at a DNP concentration of 100 ng ml^{-1} (measured as DNP-lysine, calculated from the spectrophotometric absorbance of DNP-*I-CSA at 360 nm).

Calculation of Antigen-Binding Capacity

The radioactive count in each tube was corrected by subtracting the mean count obtained from the ammonium sulphate control tubes. This subtraction is intended as a correction for non-specific binding of antigen, precipitation of denatured antigen, incomplete washing of precipitates and background radiation. The corrected count for each test sample is then divided by the similarly corrected TCA control count, to give the proportion of antigen bound. An estimate of antigen-binding capacity, ABC_{30}, was obtained from each of the eight tubes representing any one antiserum using the formula

$$ABC_{30} = -1.89\ CD \log_{10}(1-B) \qquad \text{(McKay, 1971)},$$

where C = antigen concentration, D = dilution factor of test serum and B = proportion of antigen bound. The final stage in the calculation was to obtain a mean of the eight estimates for each serum. It was necessary to use a weighted mean, since the graph of bound antigen concentration against $\log_{10}D$ (dilution factor) has a sigmoid shape, which means that where a very high or very low proportion of antigen is bound, any experimental errors can have a very large effect. The formula for calculating a weighted mean is, by definition,

$$\text{Mean (ABC)} = \frac{\Sigma(W \times ABC)}{\Sigma W}, \text{ where W is the weighting factor.}$$

The weighting factors used were calculated from the formula

$$\frac{1}{W} = 400 \left(\frac{B}{1-B}\right)^2 + 84 \frac{B}{1-B} + 40 \quad \text{(McKay, unpublished)},$$

which was a compromise between a formula derived from statistical arguments and an empirical formula which gives an imitation of the results obtained by the graphical interpolation method used by Farr.

All the above calculations were carried out using a programmable calculator (Hewlett Packard, 9100 B).

15. DETERMINATION OF THE AVIDITY OF CHICKEN ANTI-HSA ANTIBODIES

The measurement of avidity by an ammonium sulphate precipitation method enabled the effect of control mechanisms upon the avidity of circulating antibodies in the chicken to be investigated. The technique employed was based upon that recommended by Steward and Petty (1972).

Theory

The reaction between plurivalent antigen and antibody can be very complex, in that many molecular species of different structures and sizes may be formed. In order to obtain a quantitative measurement of avidity it was therefore necessary to apply certain simplifying assumptions and conditions:

(a) The antigen is a homogeneous population of molecules.

(b) The sites for combination on both the antigen and the antibody act independently of all other sites on the same molecule.

(c) Under the conditions of our measurements, the antigen is always present in excess, so that most of it remains free, some is bound to antibody at a single determinant, and the amount bound to antibody by two or more determinants can be neglected.

(d) It must also be assumed in the first instance that all the antibody combining sites have the same affinity for the antigen.

Under these conditions it is possible to represent the antigen-antibody reaction by the equation

$$Ab_s + Ag \rightleftharpoons Ab_sAg$$

where the symbol Ab_s is used to represent one antibody combining site,

Ag is used to represent a whole antigen molecule,

and Ab_sAg is used to represent a complex formed by one antigen molecule and one antibody combining site. This complex will, of course, be covalently attached to at least one other Ab_s or Ab_sAg unit, but this does not invalidate the argument. Under the four conditions imposed above, the law of mass action can be applied to the reaction as written, and the association constant K can be expressed in terms of the equilibrium concentrations of the reactants and product as follows:

$$K = \frac{[Ab_sAg]}{[Ab_s][Ag]} \qquad \text{(equation 1).}$$

It should be borne in mind that the association constant defined in this way does not depend solely on the structures of the antigenic

determinant and antibody combining site, but will increase with increasing numbers of similar determinants on the antigen molecule.

Let us now introduce the symbols [c] for the molar concentration of free antigen and [b] for the molar concentration of bound antigen, and $[Ab_t]$ for the total molar concentration of antibody combining sites (i.e. bound plus free).

Since $[c] = [Ag]$,

and $[b] = [Ab_sAg]$,

and $[Ab_t] = [Ab_s] + [Ab_sAg]$.

We can write $[Ab_s] = [Ab_t] - [b]$,

and rewrite equation 1 in the new notation:

$$K = \frac{[b]}{([Ab_t] - [b])\,[c]} .$$

Multiplying each side by $\frac{[Ab_t] - [b]}{[b]\,[Ab_t]\,K}$ gives

$$\frac{1}{[b]} - \frac{1}{[Ab_t]} = \frac{1}{[Ab_t]\,K[c]},$$

i.e. $$\frac{1}{[b]} = \frac{1}{[Ab_t]\,K} \cdot \frac{1}{[c]} + \frac{1}{[Ab_t]} .$$

This is now in the form of the equation for a straight line, $y = mx + c$. Thus if we plot $1/[b]$ against $1/[c]$ we should obtain a straight line with gradient $1/[Ab_t]\,K$ and intercept $1/[Ab_t]$ on the $1/[b]$ axis. If the gradient and intercept are determined for a set of data points it should be possible to calculate $[Ab_t]$ and K.

In practice, there is usually a deviation from the expected straight line by virtue of the heterogeneity of antibody avidities. However, this deviation may be accounted for by assuming that the

heterogeneity of avidity can be described by a Sipsian distribution function (Sips, 1948; Klotz, 1953). Thus, for binding by a large variety of sites, the mass action equation can be replaced by the Freundlich isotherm as modified by Sips (1948). Sips's equation (number 10) becomes in our notation :

$$\theta = \frac{(K_R[c])^a}{1 + (K_R[c])^a}$$

where K_R = the geometric mean association constant, commonly called the relative avidity since in practice in immunology its measurement is usually not absolute, and is useful only for comparing different sera.

θ = the proportion (mole fraction) of antibody combining sites occupied at equilibrium.

a = Sips's index of heterogeneity.

It would, strictly, be valid to use this equation only for univalent haptens, or only where conditions (a), (b) and (c) listed previously apply. Steward and Petty (1972) however have applied it to reactions involving multivalent antigens, and have used it in preference to the Langmuir adsorption isotherm.

Thus, when half the total antibody combining sites are occupied, i.e. $\theta = {}^1/_2$

then $2(K_R[c])^a = 1 + (K_R[c])^a$

i.e. $(K_R[c])^a = 1$

therefore $K_R[c] = 1$

i.e. $K_R = \frac{1}{[c]}$.

Thus the avidity K_R is equal to the reciprocal of the free antigen concentration, $1/[c]$, under those conditions where half of the total antibody combining sites are bound to antigen. This equation was used to calculate all the K_R values reported in this thesis.

Experimental procedure

Sets of six tubes were used for each antiserum being tested, each tube containing 50 µl of the undiluted test serum. To each of the set of six tubes the required amount of antigen was added, always in a volume of 50 µl. Six different concentrations of antigen were used, made from stock solutions of HSA containing 250, 500, 1000, 1500, 2000 and 2500 µg of unlabelled HSA per ml, and each of these stock solutions contained the same small amount of radio-iodinated HSA (the slight alteration in antigen concentration by addition of radio-iodinated HSA was taken into consideration when analysing the experimental results).

After mixing and overnight equilibration of the test samples, 0.2 ml of 60% saturated ammonium sulphate was added to each tube. Globulin precipitates were spun down by centrifugation at 850 x g for 30 minutes, washed in 0.5 ml 40% saturated ammonium sulphate and centrifuged as before. The final precipitates were each suspended in 0.5 ml PBS and their radioactivity was measured on an automatic gamma counter.

Standard controls were obtained in the following way:

(1) The positive controls consisted of 50 µl samples of undiluted normal chicken serum mixed together with 50 µl of antigen at each of

the different concentrations and with 0.1 ml unlabelled HSA (1 mg ml^{-1}) - this last addition was found to be necessary if all the protein-bound radioactivity in antigen solutions of low concentration was to be precipitated. To each positive control was added 0.2 ml 10% TCA and the resulting precipitates were centrifuged, washed in 10% TCA and finally suspended in 0.5 ml PBS. These positive controls indicated the radioactive counts which would be obtained if 100% of the added antigen was bound by antibody.

(2) The negative controls consisted of 50 µl samples of undiluted normal chicken serum mixed together with 50 µl of antigen at each of the different concentrations. Globulin fractions were precipitated in each negative control tube by the addition of 0.2 ml of 60% saturated ammonium sulphate (as for the test samples). These negative controls enabled any non-specific binding and/or precipitation of protein-bound radioactivity to be accounted for, and indicated the radioactive counts which would be obtained if none of the antigen was bound specifically by antibody.

All the above controls were performed in duplicate as a precaution against experimental errors.

Calculation

The first step in the calculation of avidity was to determine the concentrations of bound, [b], and free, [c], antigen from the radioactive counts measured for each test.

Let P = mean radioactivity for the two positive control tubes at a given antigen concentration, i.e. cpm equivalent to 100%

binding of added antigen by antibody.

N = mean radioactivity for the two negative control tubes at a given antigen concentration, i.e. cpm equivalent to none of the added antigen being bound specifically by antibody.

T = radioactivity in the final precipitate of each test sample.

$[Ag_t]$ = total concentration of antigen in each 0.1 ml antigen-antiserum reaction mixture.

$[b]$ = bound antigen concentration.

$[c]$ = free antigen concentration.

Therefore, the radioactivity corresponding to specific binding of antigen by antibody in a test sample (i.e. corrected for non-specific binding, precipitation of denatured antigen etc.) is equal to $T - N$; the radioactivity equivalent to 100% binding of antigen by antibody (represented by the positive controls) is equal to $P - N$. Thus the proportion of antigen added to each tube which is bound by antibody is:- $\frac{T - N}{P - N}$. Therefore, at a given concentration of antigen, $[Ag_t]$, the actual concentration of antigen added to each tube which is bound by antibody is given by $[b] = \left(\frac{T - N}{P - N} \right) \times [Ag_t]$. Similarly, the concentration of free antigen in each tube after equilibration can be calculated; $[c] = \left(\frac{P - N}{P} \times [Ag_t] \right) - [b]$.

The second step in the calculation of avidity was to make use of the values of $[b]$ and $[c]$ (six sets for each antiserum tested) in order to calculate $[Ab_t]$ and K_R.

From the equation $\frac{1}{[b]} = \frac{1}{[Ab_t]K} \cdot \frac{1}{[c]} + \frac{1}{[Ab_t]}$ it can be seen that $[Ab_t]$ may be determined by plotting $1/[b]$ against $1/[c]$ and measuring

the intercept, $1/[Ab_t]$ on the $1/[b]$ axis. The value of K_R was then determined using the relationship $K_R = 1/[c]$ when half of the total antibody combining sites are occupied by antigen; i.e. by noting from the graph the value of $1/[c]$ at the point where $[b] = [Ab_t]/2$.

Having once determined $[Ab_t]$, the next logical step would be to draw a smooth curve through the points on the $1/[b]$ against $1/[c]$ graph and find the point on the curve at which $1/[b] = 2/[Ab_t]$. The abscissa $1/[c]$ of this point is equal to K_R. However, a study of all of the graphs drawn for determining avidities revealed no systematic tendency for them to be concave down (as would be the case when applying the Sips equation to data obtained from the binding of antigen to a heterogeneous population of antibodies). If anything the reverse was true, they sometimes appeared to be linear or were very frequently concave upwards, and this latter situation particularly can be attributed only to experimental errors. It was therefore thought best to treat all the graphs as linear, and this meant that K_R could be calculated from the intercept $1/[Ab_t]$ and the gradient, $1/K_R[Ab_t]$;

i.e. K_R = intercept / gradient.

On the first occasion when avidities were determined, experimental data was processed in two ways:

(1) Using the Hewlett-Packard 9100B programmable calculator; this was programmed to determine the equation of the straight line of best fit for each set of data points. The best fit was determined by minimising the sum of the squares of the deviations of the data points

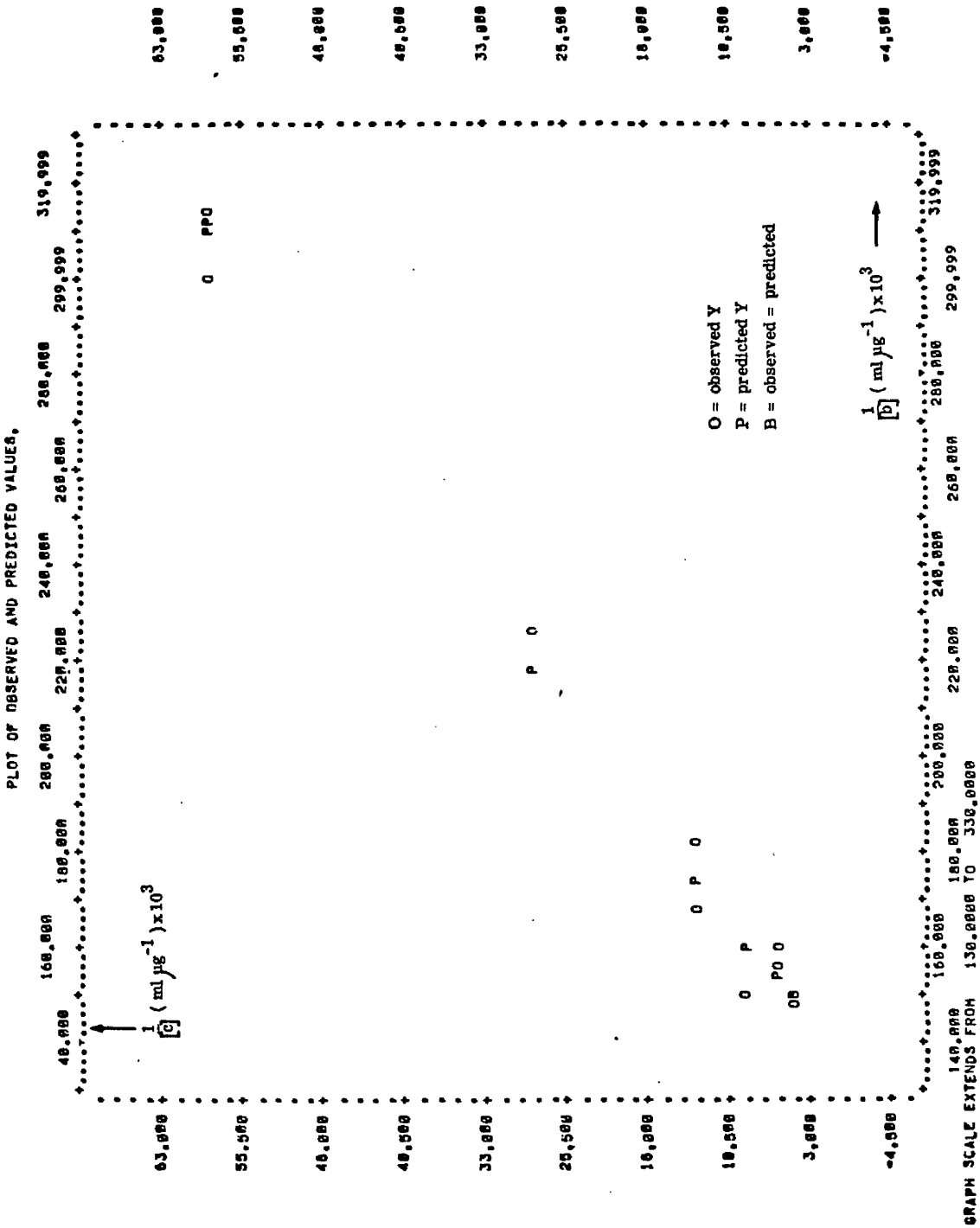


FIG. 10. Graphical print out from program BMDϕ5R showing the equation $\frac{1}{[\bar{b}]} = \frac{1}{K[\bar{c}]} \cdot \frac{1}{[\bar{Ab}_t]} + \frac{1}{[\bar{Ab}_t]}$ optimised by the method of least squares to fit 12 experimental values of $\frac{1}{[\bar{b}]}$ and $\frac{1}{[\bar{c}]}$; from this graph the values for $[\bar{Ab}_t]$ and K for a single serum were obtained.

from the line, the deviations being measured in the $1/[b]$ direction. From the best fitting straight line the values of the gradient, m, and of the intercept on the $1/[b]$ axis, $1/[Ab_t]$, were calculated.

$$\text{i.e. gradient, } m = \frac{\sum_{i=1}^{n} (X_i - \bar{X})(Y_i - \bar{Y})}{\sum_{i=1}^{n} (X_i - \bar{X})^2}$$

The intercept on the $1/[b]$ axis $= 1/[Ab_t] = \bar{Y} - m\bar{X}$.
The correlation coefficient, r, was also calculated - and this reflected how good the straight line "fit" was ($0 \leqq r \leqq 1$).

(2) Using the IBM 350/155 Computer (Edinburgh Regional Computing Centre). The treatment of results was essentially the same, but the best straight line fit was obtained using a programme for polynomial regression (program BMDØ5R, cited by Dixon, 1971). This method, although the final results were virtually identical to those determined using the programmable calculator, had the advantage that a graph print-out showing both observed and predicted values for all the data points was obtained for each antiserum tested; a typical example is shown in Figure 10.

Once it had been shown that accurate results (compared with those calculated on the computer) could be obtained using the programmable calculator, subsequent determinations of avidity values were carried out solely on the Hewlett-Packard calculator.

16. DETERMINATION OF THE AFFINITY OF CHICKEN ANTI-DNP ANTIBODIES USING EQUILIBRIUM DIALYSIS

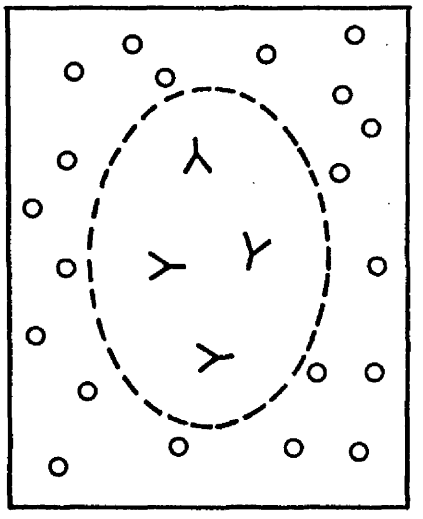
Equilibrium dialysis was used to measure the affinity of

chicken anti-DNP antibodies for the dialysable ligand DNP-ε-aminocaproic acid; the technique employed was adapted from the methods of Kabat and Mayer (1961), Eisen (1964c) and Pinckard and Weir (1973).

Theory

The technique of equilibrium dialysis makes use of the facility with which low molecular weight hapten molecules are able to pass freely from one side of a dialysis membrane to the other side, whilst larger molecules (MW > 1000 daltons, e.g. antibody molecules) cannot do so and are therefore confined to one side of the dialysis membrane. The principle of the method is illustrated in Figures 11 and 12.

The dialysis system is set up in such a way that solutions of the antibody (either whole antiserum, a crude globulin fraction or else a purified antibody preparation) and the hapten are effectively in two different compartments separated by a dialysis membrane (Figure 11). Usually the antibody is sealed inside a small dialysis sac which in turn is placed inside a sealed bottle containing a large volume of hapten solution. The hapten is then able to pass freely across the dialysis membrane and at equilibrium the concentration of free hapten on either side of the membrane (e.g. inside and outside the dialysis sac) will be the same; in addition, some hapten molecules which have crossed the dialysis membrane will be bound specifically by antibody (Figure 11(2) and Figure 12(2)) and will therefore play no part in establishing the equilibrium of free hapten across the membrane.



(1). Antibody is contained within a sealed dialysis compartment which in turn is immersed in a large volume of hapten solution at a given concentration.

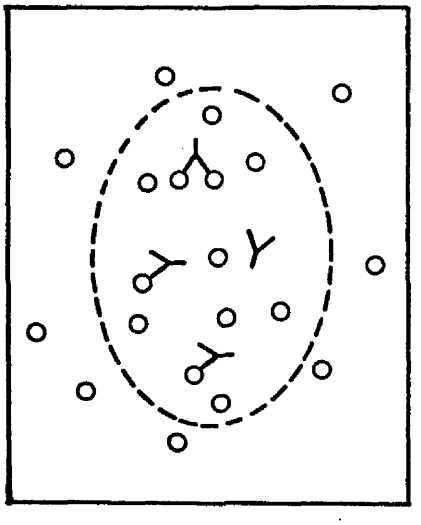
o Free hapten molecule

Hapten molecule bound to antibody

Y Antibody molecule

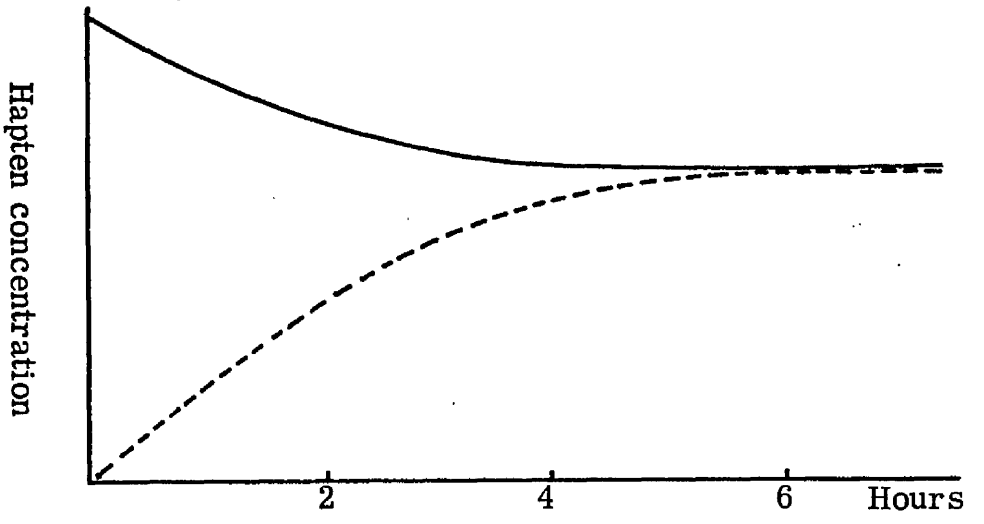
---- Dialysis membrane

EQUILIBRATION

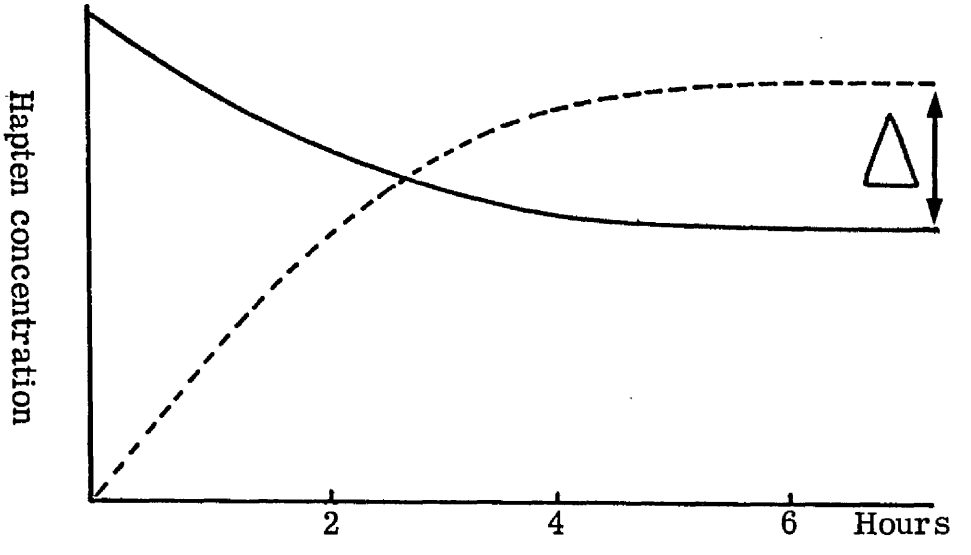


(2). After equilibration, free hapten is present in equal concentrations on both sides of the dialysis membrane; in addition, some hapten is specifically bound to antibody inside the dialysis compartment over and above the equilibrium concentration of free hapten and this is directly related to the affinity of the antibody for the hapten.

FIG. 11. Representation of equilibrium dialysis in diagrammatic form.



(1). Attainment of equilibrium in a system containing no antibody (e.g. negative control with non-immune serum), represented by the respective total concentrations of hapten outside (large volume, ———) and inside (small volume, -----) the dialysis compartment.



(2). Attainment of equilibrium in a system containing antibody represented by the respective total concentrations of hapten outside (large volume, ———) and inside (small volume, -----) the dialysis compartment. △ = the concentration of hapten specifically bound to antibody.

FIG. 12. Graphical representation of equilibrium dialysis.

The interaction of one antibody combining site (represented as Ab) with one hapten molecule (represented as H) may be described in the form of a stoichiometric equation:

$$Ab + H \rightleftharpoons AbH.$$

By determining the total and free hapten concentrations in the reaction mixture (the latter being inferred from the hapten concentration outside the dialysis sac) the concentration of bound hapten can be found by subtraction. Thus there is only one other quantity, namely the free antibody concentration, which has to be measured in order to find K, the association constant for the reaction described previously. When the reaction mixture has reached equilibrium

$$K = \frac{[AbH]}{[Ab][H]}$$

where $[Ab]$ = concentration of free antibody combining sites.

$[H]$ = concentration of free hapten.

$[AbH]$ = concentration of bound hapten.

= concentration of hapten-occupied combining sites.

If we introduce the symbol $[Ab_t]$ to represent the <u>total</u> concentration of antibody combining sites, i.e. $[Ab] + [AbH]$, then we can rewrite the formula for K in the form of the Langmuir adsorption isotherm:

$$\frac{1}{[AbH]} = \frac{1}{K[Ab_t]} \cdot \frac{1}{[H]} + \frac{1}{[Ab_t]} .$$

Thus a plot of $1/[AbH]$ against $1/[H]$ should give a straight line with gradient $1/K[Ab_t]$ and intercept $1/[Ab_t]$ on the $1/[AbH]$ axis. The values of K and $[Ab_t]$ can therefore be found from the gradient and intercept.

Nisonoff and Pressman (1958) have found that in practice such a graph is seldom linear, probably owing to heterogeneity of the antibody population, and have therefore proposed that Sips's isotherm (Sips, 1948) be used instead. Sips's equation (Equation 10; Sips, 1948), when written in a form analogous to the Langmuir isotherm above, is

$$\frac{1}{[AbH]} = \frac{1}{[Ab_t]K_o^a} \cdot \frac{1}{[H]^a} + \frac{1}{[Ab_t]} \quad .$$

Thus if $1/[AbH]$ is plotted against $1/[H]^a$, using a constant amount of antiserum and various amounts of hapten, this should give a straight line with gradient $1/[Ab_t]K_o^a$ and intercept $1/[Ab_t]$. The value of a must be found by trial and error, choosing that value which gives the straightest line possible. K_o and $[Ab_t]$ may then be calculated from the gradient and intercept.

An alternative method, which was used in this thesis for deriving K_o and a from experimental data, is shown below. It does not involve determination of a by trial and error, and it has the advantage that a very wide range of hapten concentrations can be used for the one experiment without causing clustering of points at one end of the graph. Sips's equation may be rearranged thus:

$$[Ab_t] = \left\{ 1 + (K_o[H])^{-a} \right\} . \quad [AbH] .$$

If this equation is now applied to two (hypothetical) extremes of hapten concentration .... namely [H] is either very high or else very low, then,

(1) At very high values of [H] : $(K_o[H])^{-a} \ll 1$, therefore

$$[Ab_t] = [AbH].$$

(2) At very low values of [H] : $(K_o[H])^{-a} \gg 1$, therefore

$$[Ab_t] \simeq (K_o[H])^{-a} \cdot [AbH] \text{ , or } [AbH] = [Ab_t] K_o^{\,a} \cdot [H]^a$$

Taking log of each side gives,

$$\log [AbH] = \log ([Ab_t] K_o^{\,a}) + a \log [H] .$$

Using the notation [AbH] = [b] = bound hapten concentration and [H] = [c] = free hapten concentration, the previous equation becomes

$$\log [b] = \log ([Ab_t] K_o^{\,a}) + a \log [c] .$$

These equations predict (1) that a graph of log [b] against log [c] will plateau at large values of [c], the height of the plateau giving $[Ab_t]$.

(2) the same graph at low values of [c] will be a straight line with gradient a and intercept equal to $\log([Ab_t] K_o^{\,a})$, from which K_o may be found.

Experimental procedure

Equilibrium dialysis of chicken anti-DNP antisera was carried out at seven different concentrations of the hapten DNP-ε-aminocaproic acid, each hapten solution containing a proportion of tritium-labelled hapten, $[^3H]$-DNP-EACA. The first six concentrations of hapten solution were made up to a volume of 200 ml with PBS such that there was a range of hapten solutions varying in concentration from 10^{-3} M by 10-fold dilutions to 10^{-8} M, and to each hapten solution 0.5 ml of $[^3H]$-DNP-EACA (at a concentration of 1 nmol ml^{-1})

was added; the seventh and lowest concentration of hapten was made up by adding 0.5 ml of [^{3}H]-DNP-EACA to 200 ml of PBS. The constitution and final molar concentrations of the hapten solutions for dialysis are tabulated below:

Concentration of 200 ml un-labelled hapten solutions	Volume of tritiated hapten added (1 nmol ml^{-1})	Final concentration of hapten solutions for dialysis (correct to 3 significant figures)
10^{-3} M	0.5 ml	10^{-3} M
10^{-4} M	0.5 ml	10^{-4} M
10^{-5} M	0.5 ml	10^{-5} M
10^{-6} M	0.5 ml	10^{-6} M
10^{-7} M	0.5 ml	1.02×10^{-7} M
10^{-8} M	0.5 ml	1.25×10^{-8} M
no hapten; 200 ml PBS	0.5 ml	2.50×10^{-9} M

The hapten solutions were finally transferred to plastic bottles ready for dialysis. Antibody was included in the dialysis system either as undiluted antiserum or as an ammonium sulphate precipitated globulin fraction. In either case, the antibody was dialysed against PBS before use. A measured volume of antibody (a constant amount for each test) between 0.5 and 1 ml was placed in a rigid dialysis chamber; each dialysis chamber consisted of a 1 ml auto analyser cup (Sterilin Ltd., Richmond, Surrey, England) with a dialysis membrane (made from Visking dialysis tubing, flat width 10 mm and thickness 0.32 mm, obtained from

A. Gallenkamp and Co. Ltd., Stockton-on-Tees, England) over the top secured by a polythene ring sealing the membrane over the cup.

Samples of each antiserum, together with separate controls for normal serum and PBS, were then dialysed against each of the different hapten concentrations. Polythene bottles (300 ml capacity, wide mouth with polypropylene screw caps; obtained from Macfarlane Robson Ltd., Glasgow, Scotland) containing the dialysis chambers immersed in hapten solution were rotated end over end and the solutions were allowed to equilibrate in this way for at least 48 hours at 4°.

The dialysis chambers were then removed from the polythene bottles and a 0.2 ml sample of dialysate was extracted from each chamber; this aliquot was made up to 0.5 ml with PBS and then mixed with 5 ml of scintillation fluid in a screw-capped scintillation vial. The scintillation fluid used was prepared by dissolving 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-di-2(5-phenyloxazolyl)benzene (PPO and POPOP respectively, both obtained from Nuclear Enterprises, Sighthill, Edinburgh, Scotland) in 1000 ml toluene at 50° and adding 500 ml of Triton X-100 (British Drug Houses, Poole, England). The samples were counted for 10 minutes each in a Packard Model 3320 Tri-Carb Scintillation Spectrometer. The radioactive counts for each sample were subsequently corrected for quenching by the channels ratio method.

Calculation

The first step in calculating the affinity constant for an

antiserum was to determine the bound, [b], and free, [c], hapten concentrations from the equilibrium dialysis data.

Let T = ^{3}H cpm for test serum <u>after</u> dialysis, corrected for quenching and background.

N = ^{3}H cpm for normal serum <u>after</u> dialysis, corrected for quenching and background.

P = ^{3}H cpm for PBS control <u>after</u> dialysis, corrected for quenching and background.

S = ^{3}H cpm for a hapten solution <u>before</u> dialysis, corrected for quenching and background.

$[H_t]$ = molar concentration of hapten <u>before</u> dialysis.

Then, the molar concentration of bound hapten, $[b] = \frac{T - N}{S} \times [H_t]$ and the molar concentration of free hapten, $[c] = \frac{P}{S} \times [H_t]$. (Table 3 shows typical equilibrium dialysis data used for calculating [b] and [c]).

The equation $\log[b] = \log([Ab_t]K_o^a) + a \log[c]$ predicts a linear relationship between log[b] and log[c] at low hapten concentrations. At high concentrations of hapten (i.e. where hapten is saturating the combining sites on all antibody molecules), then the relationship $\log[b] = \log[Ab_t]$ is applicable. Thus, from the value of log [b] corresponding to the plateau of the graph it was possible to find $[Ab_t]$; likewise, from the straight line part of the graph, it was possible to calculate the index of heterogeneity, a (= gradient). The intercept on the log[b] axis, obtained by extrapolating the straight line portion of the graph, is equal to $\log([Ab_t]K_o^a)$. K_o can be

TABLE 3. Information typically obtained from equilibrium dialysis of chicken anti-DNP antisera

Counts/10' in hapten solutions before dialysis	Molar concentration of hapten before dialysis	Counts/10' in hapten solutions after dialysis	Counts/10' in PBS control after dialysis	Molar concentration of hapten after dialysis	Counts/10' in non-immune globulin control	Test sera from immunised birds; ^3H counts/10' after dialysis						
						No.976	978	979	980	984	986	
2,444	10^{-3}	2,010	1,969	0.81×10^{-3}	2,115	2,027	2,041	2,185	2,224	2,074	2,064	2,0
1,897	10^{-4}	1,902	1,867	0.99×10^{-4}	2,093	2,276	2,330	2,303	2,237	2,163	2,246	2,1
2,179	10^{-5}	1,832	1,766	0.83×10^{-5}	2,341	3,004	3,482	3,894	2,537	2,832	3,652	2,2
2,162	10^{-6}	1,587	1,610	0.74×10^{-6}	2,262	6,255	8,498	11,159	2,768	5,893	10,730	2,1
2,162	1.025×10^{-7}	1,255	1,186	0.56×10^{-7}	2,364	9,131	10,179	11,711	3,395	9,691	3,227	2,6
2,213	1.25×10^{-8}	957	1,111	0.63×10^{-8}	2,857	9,826	10,502	9,652	4,181	10,726	10,642	3,1
2,142	2.5×10^{-9}	1,085	1,397	1.63×10^{-9}	3,674	11,831	11,706	12,094	5,355	12,014	13,395	4,1
	Affinity constant, K_o (litres mole^{-1})						6.7×10^5	5.7×10^5	1.1×10^6	1.1×10^3	6.7×10^3	4.7×10^5	9.9
	Index of heterogeneity, a						0.95	0.97	1.0	0.73	0.32	0.95	0.7

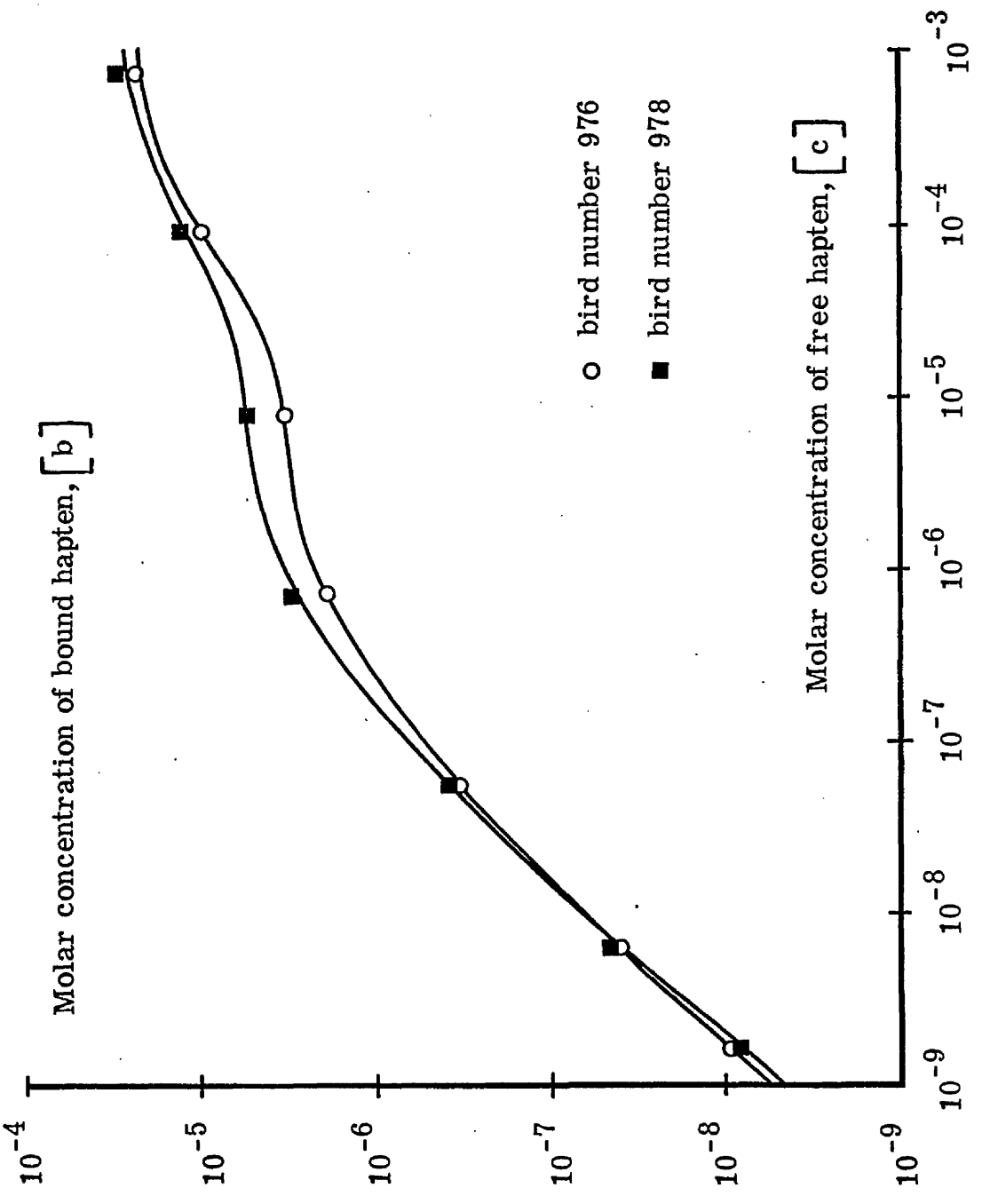


FIG. 13. Typical plot of data from equilibrium dialysis of anti-DNP antibodies from two individual birds, using the ligand DNP-EACA.

calculated from this value, since $[Ab_t]$ and a have already been determined. In practice, since a programmable calculator was available, it was found more convenient to calculate the intercept from two points lying on the linear portion of the graph, rather than extrapolating graphically. A programme was written for a Hewlett-Packard 9100B calculator which would compute K_o and a when supplied with the value of $[Ab_t]$ (obtained from the plateau level) and the co-ordinates (x_1, y_1) and (x_2, y_2) of two points lying on the linear part of the graph. The calculation was as follows:

$$a = \text{gradient} = (y_2 - y_1) / (x_2 - x_1).$$

$$\log [Ab_t]K_o^{\,a} = \text{intercept} = y_1 - ax_1,$$

$$\text{therefore} \quad K_o = \left(\frac{10^{y_1 - ax_1}}{[Ab_t]} \right)^{1/a}.$$

Figure 13 shows two typical graphs of log [b] plotted against log [c] from which data for the foregoing calculations could be obtained. These graphs were constructed from information contained in Table 3.

17. FLUORESCENT ANTIBODY TECHNIQUE AND DETECTION OF ANTIBODY-SECRETING CELLS IN TISSUE SECTIONS

The fluorescent antibody technique was used for the detection and enumeration of specific anti-HSA-producing plasma cells in the spleens of immune birds. The techniques employed are described below.

Preparation of fluorescein-conjugated antibodies

Antiserum from rabbits immunised with HSA was labelled with fluorescein isothiocyanate (FITC; obtained from British Drug Houses Ltd., Poole, England) using the procedure described by White, French and Stark (1970), which was itself based on the method of Coons, Creech, Jones and Berliner (1942); the technique may be summarised as follows.

The globulins from 10 ml of rabbit anti-HSA antiserum were precipitated by the slow addition of saturated ammonium sulphate, previously cooled to 4°, such that the final mixture was 40% saturated in ammonium sulphate. The mixture was stirred mechanically for 30 minutes at 4° and the precipitate was then sedimented by centrifugation; following this the precipitate was dissolved in PBS to the same volume as the original volume of serum, and was re-precipitated as before by addition of saturated ammonium sulphate. This final precipitate was sedimented, dialysed exhaustively against PBS, and adjusted to a protein concentration of between 10 and 12 mg ml^{-1}. A volume of 8.5 ml of the resulting globulin solution (containing approximately 100 mg protein *in toto*) was labelled with fluorescein by adding 1.5 ml of fluorescein isothiocyanate solution (at 1 mg ml^{-1} in 0.5 M sodium carbonate/bicarbonate buffer, pH 9.0). The conjugation was carried out for 12-18 hours at 4° with mechanical stirring. Labelled protein was separated from un-attached fluorochrome by gel filtration on a 30 cm x 1 cm column of Sephadex G25 equilibrated with PBS. Protein molecules carrying an excess of fluorescein (and therefore, by virtue of their high net negative charge, having a tendency to be absorbed non-specifically

onto tissue sections) were selectively removed by running the conjugate through a 20 cm x 1 cm column of diethylaminoethyl-cellulose (DEAE-cellulose, in the form of DE22 obtained from Whatman Biochemicals Ltd., Maidstone, Kent, England) and eluting the utilisable fluorescent antibody with PBS - unsuitable material remaining on the ion-exchanger.

Preparation and immunofluorescent staining of chicken tissues

Chickens were killed by an overdose of intravenous sodium pentobarbitone (as described on page 78). The spleens of birds were excised and cut into two or three pieces which were lodged on the walls of glass test tubes; these tubes were sealed with a rubber bung and rapidly frozen by immersion in an acetone/solid CO_2 freezing mixture at -70°. Sections 5 µm thick were cut in a cryostat at -20°, placed on glass microscope slides, melted and dried in a stream of warm air and fixed in absolute methanol at 20° - 24° for 15 minutes. After being rapidly dried in a current of warm air, the sections were stained in the following manner.

Detection of plasma cells containing anti-HSA

The presence of antibody was shown using the "sandwich" technique of Coons, Leduc and Connolly (1955). A solution of HSA (2 mg ml^{-1} in saline) was applied to the spleen sections which were then left for 30 minutes. Following this, the sections were washed thoroughly in three changes of PBS and then stained with fluorescein-labelled rabbit anti-HSA (1 drop) for a further 30 minutes. Stained sections were finally washed in three changes of PBS and mounted in 30% glycerol in PBS under cover-slips which were sealed with nail

varnish. This technique will also stain any antigen present in the section as well as antibody; however, by comparison of two adjacent sections, one stained by the "sandwich" technique and the other stained by the direct method (i.e. omitting the "sandwich" layer of antigen and therefore staining directly and exclusively for HSA in the sections) antibody can be differentiated from antigen by its presence only in the section stained by the two layers of the "sandwich" technique.

Fluorescence microscopy

The tissue sections stained either directly or by the "sandwich" technique were examined microscopically using a Wild M 20 monocular microscope (Wild, Heerbrugge, Germany), fitted with dry and oil-immersion objectives and a bi-reflecting dark-ground condenser. The light source for fluorescence microscopy was an Osram HBO 200 high-pressure mercury arc contained within a Wild Universal Microscope Lamphouse. The exciter filters were BG12 (transmits ultraviolet and blue light) and BG38 (heat filter) (Schott and Genossen, Mainz, Germany); the barrier filter was a Schott OG1 (absorbs ultraviolet and blue light; transmits red, orange, yellow and green). A conventional tungsten lamp, for white light observations, was built into the base of the microscope and could be brought into use by swinging out a mirror transmitting the light from the mercury arc to the condenser. Using the fluorescence arrangement, plasma cells containing antibody to HSA appeared as oval cells with an unstained eccentric nucleus and brightly fluorescent cytoplasm.

The calculation of the number of specific anti-HSA plasma cells per unit area of spleen section was done by counting the total number of specific plasma cells, t, in a given number of different viewing fields, n, of the splenic red pulp. When using the X54 oil-immersion objective lens in combination with a X6 eyepiece, the diameter, d, of the viewing field (as judged as accurately as possible from the gradations on a haemocytometer) was 0.30 mm. Therefore, the area of each viewing field is $\pi(d/2)^2$ and the total area of tissue section examined is $\pi(d/2)^2 \times n$; accordingly, the number of specific plasma cells in a unit area of spleen section is equal to

$$\frac{t}{\pi(d/2)^2 \times n} = \frac{t}{\pi(0.15)^2 \times n} \quad mm^{-2} .$$

It was considered that the number of specific plasma cells per unit area of a tissue section of uniform thickness was a more accurate way of expressing the results rather than as the number of specific plasma cells per unit volume; since each section may be assumed to be approximately 5 μm thick, and if a chicken plasma cell has a diameter of at least 8 μm (Lucas and Jamroz, 1961), then the tissue section will contain no complete plasma cells - only parts of plasma cells. When calculating the number of plasma cells per unit volume however, these sectioned plasma cells could not reasonably be regarded as complete plasma cells, as this might inflate the final cell count. The measurement of plasma cell population density as the number of plasma cells per unit area of tissue section was therefore preferred.

18. IMMUNODIFFUSION

Immunodiffusion was used for the qualitative assessment of sheep, rabbit and chicken antisera to protein antigens; the technique used was one of double radial diffusion as described by Ouchterlony and Nilsson (1973), and is summarised below.

A 1.5% solution of agar (Special Agar-Noble, Difco Laboratories, Detroit, Michigan, U.S.A.) in barbitone buffer (0.05 M, pH 8.5) was melted and spread in an even layer 1 mm thick over the bottom of a plastic Petri dish (85 mm diameter x 15 mm). After the agar had set, up to six circular wells (diameter 1.2 mm) spaced equally on an imaginary circle (diameter 10 mm) around a central well were cut in the agar. The reference antigen solution was placed in the central well and the test solutions were placed in the surrounding wells. The gel was then left at room temperature in a damp chamber for 24 - 48 hours to allow diffusion and lines of precipitate to develop; an example is shown in Figure 14a.

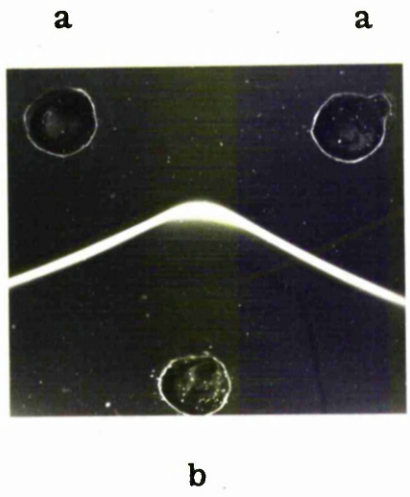
For testing chicken antisera the barbitone buffer used contained 8% NaCl. This complied with the findings of Hersh and Benedict (1966) that precipitation of chicken IgG antibody by antigen at high salt concentration yields move precipitable chicken protein, especially in the zone of antigen excess, than precipitation at low salt concentration.

19. IMMUNOELECTROPHORESIS

Immunoelectrophoresis was used for the qualitative assessment

of rabbit and chicken antisera to protein antigens, and for immunoglobulin class characterisation of chicken antibodies. A microtechnique, as described by Ouchterlony and Nilsson (1973) was employed.

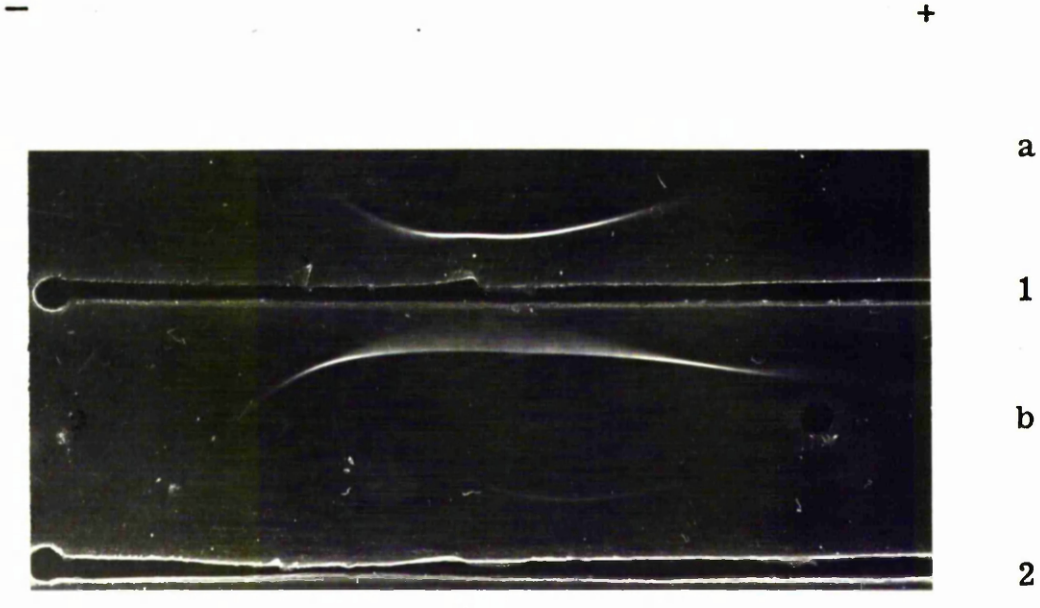
A 1.5% solution of melted agar in barbitone buffer (0.05 M, pH 8.5) was spread in an even layer (approximately 1 mm thick) over a clean glass plate, either 10 x 10 cm or 10 x 20 cm. After the agar had set, circular wells, and troughs parallel to the flow of current were cut alternately in the agar at 5 mm intervals. The wells were filled with test solution (e.g. serum, serum fractions or purified proteins) and the agar gel was connected by means of paper wicks to the cathode and anode reservoirs (containing barbitone buffer, 0.05 M, pH 8.5) on respective sides of an electrophoresis tank. A potential difference of 200 V was applied, causing a current of about 17.5 mA to flow. A marker solution containing normal serum and a small amount of bromophenol blue was electrophoresed at the same time as the test solutions; bromophenol blue adheres to the albumin in the serum and therefore moves as a discrete blue spot towards the anode. The time allowed for electrophoresis (usually about 2 hours) was judged from the position of the bromophenol blue/albumin spot, albumin being the fastest moving serum component. After electrophoresis the troughs were filled with the appropriate reagents (e.g. unispecific or multispecific antisera) and diffusion of these against the electrophoresed fractions of the test solutions was allowed to proceed for 24 - 48 hours at room temperature, in a moisturised environment. The specificity of antisera or the purity of serum fractions could be



well a: sheep anti-HSA.

well b: HSA (0.5 mg ml^{-1}).

FIG. Double diffusion of sheep anti-HSA antiserum.



well a: chicken 7S immunoglobulin.

well b: chicken serum.

trough 1: sheep anti-chicken immunoglobulin.

trough 2: rabbit anti-chicken μ chain.

FIG. 14. Immunoelectrophoresis of chicken immunoglobulins.

inferred by inspection of the precipitate arcs which developed in the agar (e.g. as shown in Figure 14b).

Staining of immunodiffusion and immunoelectrophoresis agar gels

Unprecipitated material was removed from the agar by washing the gels in saline for 24 hours. The gel was then immersed for 2 minutes in 0.5% amido black (naphthalene black) (G.T. Gurr Ltd., London, England) in methanol/glacial acetic acid (9:1 v/v). The gel was washed several times in methanol/glacial acetic acid solvent, and then in saline and water respectively. Once the gel had been dried it could be kept as a permanent record of the test.

20. TANNING AND COATING OF RED BLOOD CELLS; HAEMAGGLUTINATION TESTS

Haemagglutination was used to measure anti-erythrocyte (human) antibody titres in the serum of chickens. Sheep red blood cells which had been tanned and coated with antigen (HSA) were also prepared and used for immunising chickens. The methods for tanning and coating of red blood cells and for the haemagglutination tests followed the procedures recommended by Herbert (1973).

Tanning of red blood cells for use in haemagglutination tests

Fresh human erythrocytes were obtained by allowing heparinised venous blood mixed with $^1/_4$ of its volume of dextran (6% in saline) to stand for 30 minutes, and then aspirating the supernatant leucocyte-rich plasma from the sedimented red blood cells. The erythrocytes were washed three times in saline and were packed down each time by centrifugation at 750 x g for 10 minutes. Packed cells (0.6 ml) were

pipetted into 1 oz screw-capped glass bottles and suspended in 10 ml of saline; the erythrocytes were then sedimented by centrifugation at 750 x g for 10 minutes. The cells in each bottle were resuspended in 9.5 ml saline and to this 0.5 ml tannic acid solution (1 mg ml^{-1} in saline) was then added; the suspensions were incubated for 15 minutes at 37° in a water bath with mechanical shaker. The erythrocytes were again spun down and then resuspended in 10 ml saline containing 1% heat-inactivated normal chicken serum from which any natural anti-erythrocyte antibodies had previously been absorbed with the red blood cells to be used in the test. The tanned red blood cells were washed three times in saline containing 1% heat-inactivated absorbed normal chicken serum. The contents of each bottle were finally resuspended to a final volume of 50 ml in saline containing 1% heat-inactivated normal chicken serum - giving therefore a 1% suspension of erythrocytes (i.e. approximately 10^8 erythrocytes ml^{-1} for human red blood cells).

Coating of sheep red blood cells with HSA

Sheep erythrocytes were obtained by dextran sedimentation from whole blood exactly as described for human erythrocytes in the preceding section. After three washes in saline, 6 ml of packed cells were pipetted into large screw-capped glass bottles and suspended in 100 ml saline; the erythrocytes were then sedimented by centrifugation at 750 x g for 10 minutes. The cells were resuspended in 95 ml saline and to this was then added 5 ml tannic acid solution (1 mg ml^{-1} in saline); the suspensions were incubated for 15 minutes at 37° in a water bath with mechanical shaker. The SRBC were then washed twice in

saline and resuspended each time in 50 ml saline. To each bottle containing 50 ml suspended SRBC, 50 ml of antigen (200 mg HSA in 50 ml saline) were added and the bottles were incubated for 30 minutes at 37^{o} in a water bath with mechanical shaker. The coated SRBC were then washed three times with saline and the packed cells were finally resuspended in about two volumes of saline (10 - 12 ml) to give as nearly as possible 10^{10} HSA-coated SRBC ml^{-1}.

Haemagglutination test

Starting at a 1/10 dilution of antiserum in saline, doubling dilutions (using Takatsy microdiluting loops) were made, again with saline, into consecutive wells of plastic microtitre haemagglutination plates (Flow Laboratories, Irvine, Scotland); this gave a final range of antiserum dilutions from 1/10 to 1/5120, each in a total volume of 25 µl. To each well a 25 µl volume of a 1% suspension of tanned human red blood cells was added, and the plate was left undisturbed overnight at 4^{o}. The plates were examined the next day for agglutination of the red blood cells; for each antiserum the antibody titre was measured as the reciprocal of the highest serum dilution which gave an unequivocally positive agglutination reaction. A negative control was always included, consisting of one well containing 25 µl of the tanned human red blood cells mixed with an equal volume of saline.

EXPERIMENTAL RESULTS

Antibody characteristics have been studied and treated as being a useful reflection of the available receptors and of the responsiveness of the cells bearing them - and hence of the process of cell selection. This is especially true in the chicken, where the relatively short half lives of immunoglobulins mean that cellular events (e.g. cessation of antibody biosynthesis) are followed closely by, and readily inferred from, serum antibody levels. Primarily there are the events of cell selection and activation, which dictate the diversity of antibody specificities and the course of biosynthesis; secondary to these are various accessory mechanisms which modulate the magnitude and duration of the response once its specificity or specificities have been established.

The experimental work in this thesis explored certain aspects of the events leading to the biosynthesis of antibody as well as a number of factors which can regulate and modify the antibody response. Accordingly, the results have been categorised as follows:

Evidence on the mechanism of cell selection

I. The kinetics of antibody production in chickens in response to immunisation with proteins and with hapten-protein conjugates.

II. The affinity of anti-hapten and the avidity of anti-protein chicken antibodies.

Evidence for accessory control mechanisms

I. The effects of adjuvants upon antibody biosynthesis in chickens.

II. Antibody-mediated immunosuppression as an experimental model of negative feedback homeostasis in chickens.

EVIDENCE ON THE MECHANISM OF CELL SELECTION

I. The Kinetics of Antibody Production in Chickens in Response to Immunisation with Proteins and with Hapten-Protein Conjugates

Experiment 1. The Kinetics of Antibody Biosynthesis in Chickens in Response to Immunisation with a Single Intravenous Injection of HSA

This first experiment was carried out to investigate the kinetics of the anti-HSA antibody response in chickens following immunisation with a single intravenous injection of HSA.

If the characteristics of antibody biosynthesis are to be used as a marker of the selective pressures imposed upon receptor molecules, then obviously an overall impression of the kinetics of biosynthesis is necessary. The first few days of antibody production are particularly interesting in this context, since they reflect the processes of cell recruitment, proliferation and differentiation - presumably relatively free of the complications of negative feedback mechanisms which might be expected to come into action at a later stage. An attempt was therefore made to determine and confirm the kinetics of anti-HSA biosynthesis in the chicken and to justify the pattern of kinetics in terms of receptor selection, followed by cellular activation and clonal expansion.

Experimental procedure A group of 12 chickens (10 weeks of age) were each immunised by a single intravenous injection of 250 µg HSA

in 0.5 ml saline. The birds were bled (approximately 2 ml each) 3, 7, 12 and 21 days following primary immunisation. The serum from each blood sample was assayed for its antigen-binding capacity (ABC_{30}) by the Farr test using radio-iodinated HSA.

Results As can be seen from Table 4 and Figure 15, anti-HSA was virtually undetectable in the serum 3 days after immunisation but thereafter there was a rapid rise in the antigen-binding capacity of the serum from each bird, reaching a maximum at about 7 days after immunisation. Antibody levels fell sharply after this time with the result that 3 weeks after immunisation there was little or no anti-HSA antibody detected in the serum.

It might be assumed that the rate of reaction between antigen and an antibody-like receptor molecule on lymphoid precursor cells is similar to the rate observed for interaction between antigen and antibody molecules in vitro; i.e. once the antigen has gained access to the antibody-forming precursor cells the association between antigen and receptor, whereby selection is accomplished, takes place relatively rapidly. On this presumption the rate-limiting step in antibody biosynthesis is therefore the subsequent events of cellular proliferation and differentiation. The chickens' response to a soluble protein antigen was compatible with a rapid recruitment of antibody-forming cells which did not however manifest itself, owing to the inertia of cellular development and clonal expansion, until 4 or 5 days after immunisation.

TABLE 4. Serum anti-HSA antibody levels (ABC_{30} in μg HSA ml^{-1}) in chickens following immunisation with 250 μg HSA iv.

BIRD NUMBER	DAYS AFTER IMMUNISATION			
	3	7	12	21
399	0.61	7.31	2.53	1.40
401	0.46	11.11	3.88	1.50
425	1.00	9.54	2.58	1.80
431	0.89	11.43	3.71	2.24
407	1.23	9.21	3.70	3.68
402	0.83	8.49	2.98	1.66
411	0.97	12.20	6.38	2.36
416	0.88	8.83	3.18	2.89
418	1.13	3.86	2.04	1.28
419	1.47	7.45	3.60	1.61
421	0.89	8.10	4.72	2.88
429	0.63	10.43	3.38	1.96
MEAN (±SE)	0.92±0.08	9.00±0.65	3.56±0.33	2.11±0.22

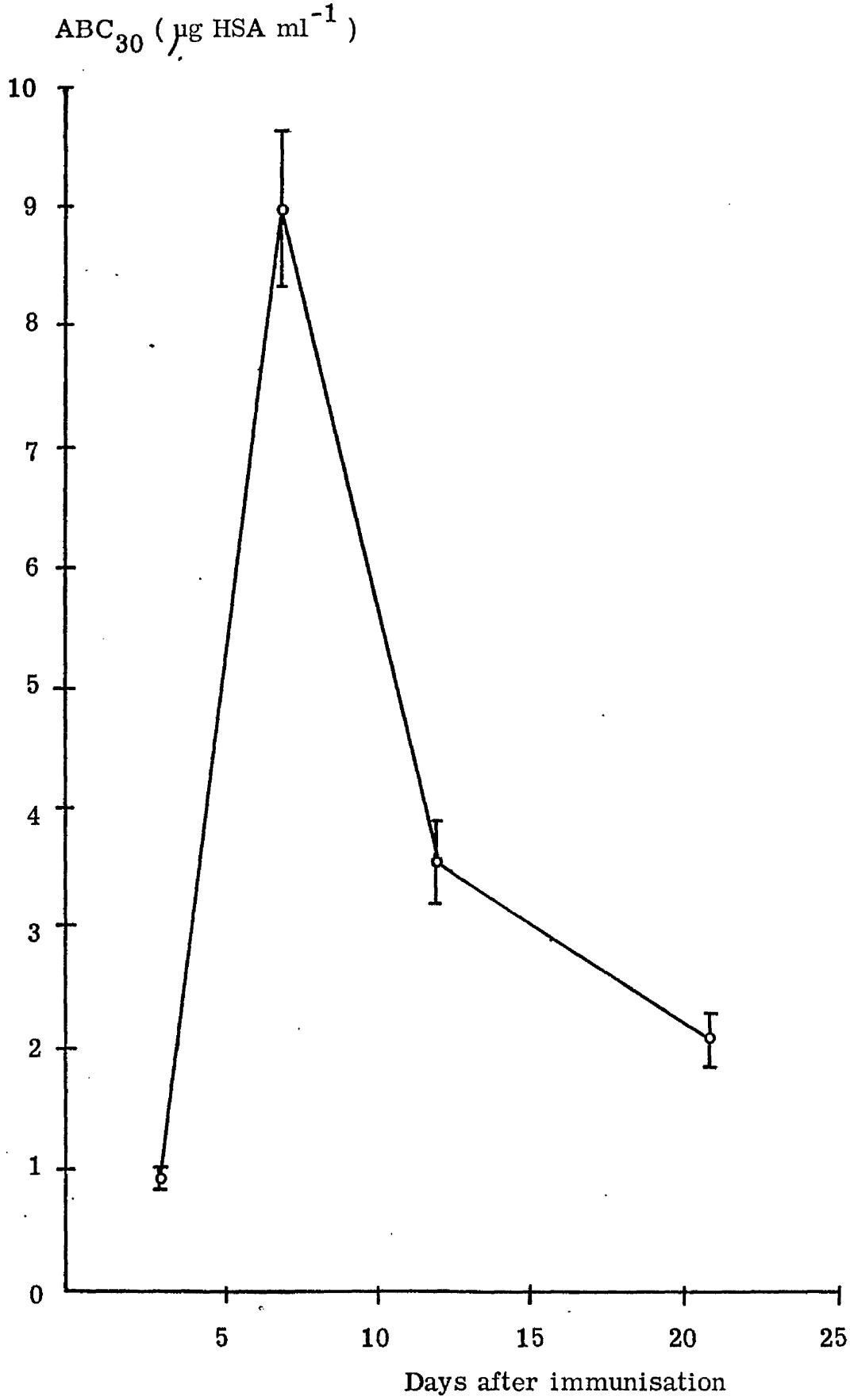


FIG. 15. Mean values (± SE) for serum anti-HSA antibody levels in chickens following immunisation with 250 µg HSA iv.

Experiment 2. The Population Density in the Chicken Spleen of Plasma Cells Secreting Specific Antibody Studied at Various Times after Immunisation with HSA

The results from the previous experiment suggested that following the administration of antigen to chickens there was a prompt selection and stimulation of precursor lymphoid cells with the subsequent production of specific antibody. The following experiment involved a closer examination of the cellular processes preceding the release of antibody molecules into the circulation, by studying the rise and fall of the plasma cell population, secreting anti-HSA, in the chicken spleen after primary immunisation.

Experimental procedure A group of 10 chickens (8 weeks of age) were each immunised by a single intravenous injection of 10 mg HSA in 1 ml saline. Individual birds were killed at intervals of 1 to 8 days following immunisation; their spleens were removed and frozen sections were prepared and then stained by the indirect "sandwich" fluorescent antibody technique to demonstrate cell-associated anti-HSA. The red pulp of each spleen section was scanned methodically under the microscope using ultra-violet illumination, and the number of positively stained (HSA-producing) plasma cells within a given number of microscope viewing fields (50 or 100) was counted.

Results The results were expressed as the number of positively stained plasma cells per square millimetre of spleen section; these values are given in Table 5 and the mean value for each bird is represented graphically in Figure 16. The method for calculating the

TABLE 5. Population density of plasma cells, expressed as the number of specific anti-HSA plasma cells observed by immunofluorescence per mm^2 of spleen section (approximate thickness 5 μm), in the spleens of chickens at various times after immunisation with 10 mg HSA iv.

DAYS AFTER IMMUNISATION	NUMBER OF BIRDS EXAMINED	TOTAL NUMBER OF PLASMA CELLS OBSERVED	NUMBER OF VIEWING FIELDS EXAMINED	NUMBER OF PLASMA CELLS PER mm^2	MEAN NUMBER OF PLASMA CELLS (±SE)
1	1	0	50	0	0
2	1	0	50	0	0
3	2	1 (223	100	31.5	
		1 (226	100	32.0	27.7
		2 (196	100	27.7	(±2.9)
		2 (138	100	19.5	
4	2	1 (554	100	78.4	
		1 (402	100	56.9	57.2
		2 (363	100	51.4	(±7.7)
		2 (298	100	42.2	
5	1	(352	100	49.8	48.4
		(332	100	47.0	(±1.4)
6	1	*			
7	1	(130	100	18.4	24.1
		(210	100	29.7	(±5.7)
8	1	18	50	5.1	5.1

* bird died

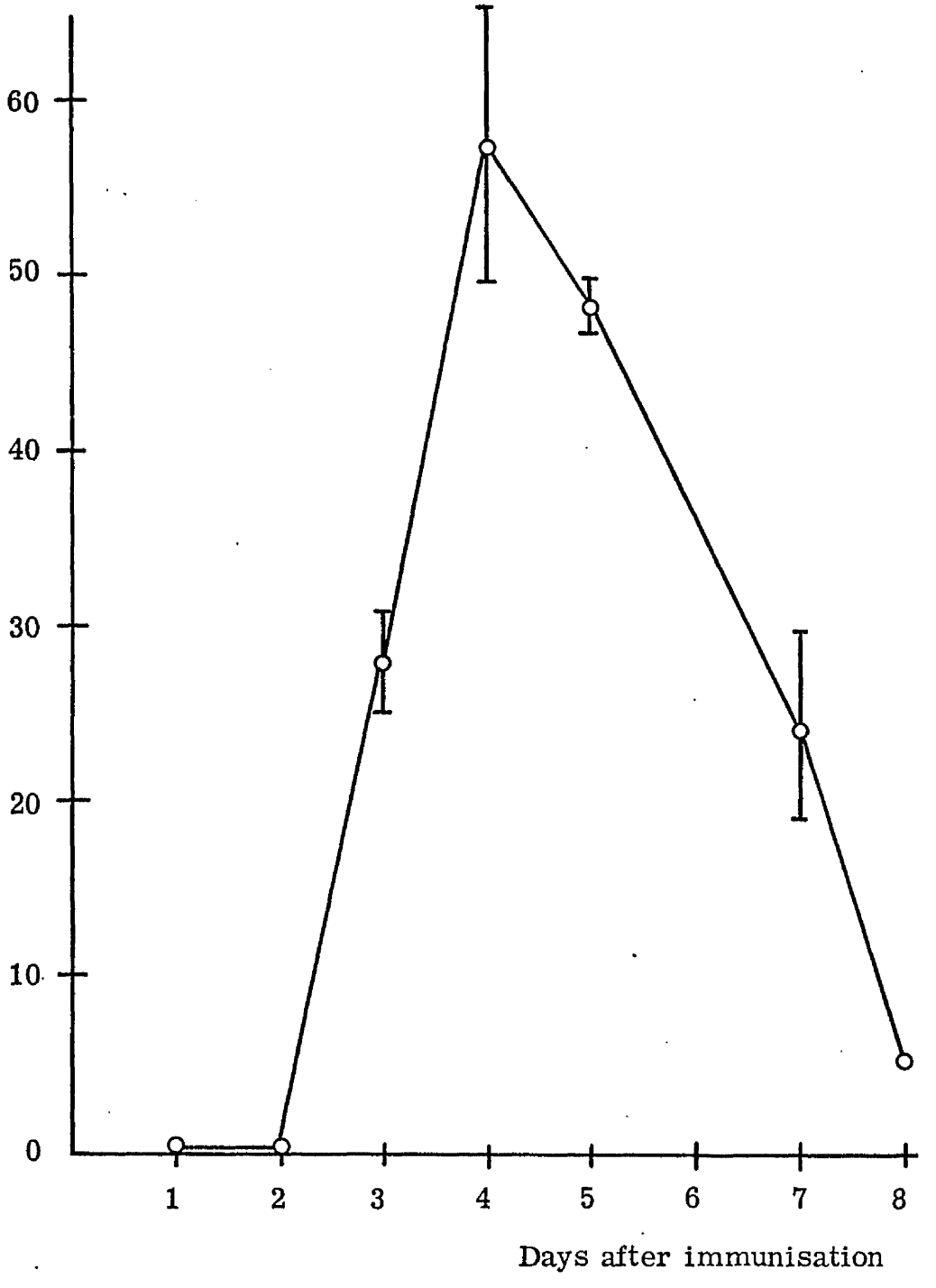


FIG. 16. Population density of plasma cells, expressed as the number of specific anti-HSA plasma cells observed by immunofluorescence per mm^2 of spleen section (approximate thickness 5 μm), in the spleens of chickens at various times after immunisation with 10 mg HSA iv.

population density of plasma cells was described on page 107.

Plasma cells secreting anti-HSA in response to immunisation with HSA were first detected in the red pulp of the chicken spleen as early as 3 days after immunisation (Table 5; Figure 16). Maximum numbers of plasma cells were found 4 and 5 days after immunisation (when the serum antibody levels were only just beginning to rise), and after 7 days (i.e. at the approximate peak of antibody levels in the circulation) the number of plasma cells had declined markedly.

The results from this experiment showed that there was a very rapid appearance and subsequent increase in the number of specific plasma cells which preceded a parallel sequence of events in serum antibody levels by approximately 2 days.

Experiment 3. The Kinetics of Antibody Biosynthesis in Chickens in Response to Immunisation with the DNP Hapten

The kinetics of antibody biosynthesis and the cellular events following immunisation with HSA have been established in the previous experiments, and the results were in agreement with the observations of other workers (e.g. White, French and Stark, 1970) who have also studied the antibody response to HSA in chickens. As the next step towards a satisfactory investigation of the affinity of anti-hapten antibodies, the kinetics of the anti-DNP response in chickens were examined. This was a preliminary study to assess the suitability of chickens as producers of anti-hapten antibody of high affinity; also to assess the importance of the physico-chemical

nature of the immunogen (e.g. the type of carrier, the epitope density) as well as the effect of carrier pre-immunisation and the importance of the age of the birds used for immunisation.

(a) The Effect of the Type of Carrier upon the Anti-Hapten Antibody Response in Chickens

It is well known that the carrier to which haptens are attached has a considerable influence upon the eventual antibody response to that hapten. In this experiment, the efficiency of different carriers, both particles and soluble proteins, for promoting biosynthesis of antibodies against the DNP group in chickens, was examined.

Experimental procedure Three groups of chickens (6 weeks of age) were each immunised as follows.

Group 1 : DNP-cellulose; a suspension of 10 mg ml^{-1}, 0.5 ml iv.

Group 2 : DNP-haemocyanin; 2 mg ml^{-1}, 0.5 ml iv.

Group 3 : DNP-HuRBC's; 2 x 10^9 cells ml^{-1}, 0.5 ml iv.

The methods of preparing the antigens used for immunising birds in Groups 1, 2 and 3 were described on pages 62, 55 and 61 respectively. For all three antigens used in this experiment it was not possible to determine the degree of hapten substitution. After immunisation, blood samples were collected at regular intervals and the serum from each was assayed for its hapten-binding capacity by the Farr test using radio-iodinated DNP-CSA.

Results The results were expressed as the hapten-binding capacity for each serum sample and are given in Table 6. In addition, the anti-HuRBC agglutination titre of antiserum obtained from each bird 7 days after immunisation is included.

An anti-hapten antibody response, as measured by a modified Farr test using radio-labelled DNP-CSA (see page 80), was not detected when using chromatographic cellulose as a carrier for the DNP group; this was inferred from the failure of serum samples taken 7 days after immunisation to bind significantly more hapten than serum taken after only 3 days. By the same criterion, the DNP hapten conjugated either to human erythrocytes or to snail haemocyanin did elicit an antibody response, albeit slight, by at least 7 days after primary immunisation (Table 6). Thus, although the human erythrocytes and snail haemocyanin were shown in a limited manner to be better carriers than cellulose, the amount of anti-DNP antibody detected was small. This could be attributed to an unsuitable method of immunisation or to an inherent inability of the birds to respond to the hapten-carrier combinations used. No attempt was made to measure antibodies to either of the carrier molecules used for immunising birds in Groups 1 and 2, but the serum from each bird was tested 7 days after immunisation for antibodies to human erythrocytes using the haemagglutination test described on page 113; this showed that birds in Group 3 (immunised with DNP-HuRBC) had all formed antibodies to the carrier erythrocytes, whereas the birds in Groups 1 and 2 (with one apparent exception) had no antibodies to human erythrocytes in their serum. It is worth noting

TABLE 6. The influence of the carrier upon the anti-hapten antibody response in chickens to a haptenic substituent (DNP). Serum levels of anti-DNP were measured by the Farr test using radio-labelled DNP-CSA and are expressed as the hapten-binding capacity of each serum sample (HBC_{30}, ng DNP-lysine ml^{-1}). In addition, the anti-HuRBC titres (expressed as the reciprocal of the highest serum dilution giving an unequivocally positive reaction) are given for serum samples collected from the birds 7 days after immunisation.

BIRD NUMBER	IMMUNISATION	DAYS AFTER IMMUNISATION 3	7	10	ANTI-HuRBC TITRES 7 DAYS AFTER IMMUNISATION
		SERUM ANTI-HAPTEN ANTIBODY LEVELS (HBC_{30}, ng DNP-lysine ml^{-1})			
652	Group I :	24	27	*	< 10
653	DNP-cellulose	16	18	16	< 10
654	5 mg iv.	13	12	*	< 10
655	Group 2 :	22	170	*	1280
656	DNP-HCN	26	140	*	< 10
657	1 mg iv.	13	97	*	< 10
658		31	1294	*	< 10
659	Group 3 :	15	94	19	2560
660	DNP-HuRBC	8	52	*	2560
661	10^9 iv.	16	35	43	2560
662		15	39	*	1280
663		18	67	37	2560
664		11	38	19	1280

* birds died of coccidiosis.

that during the course of the experiment many of the birds were suffering from coccidiosis, which was apparent from post-mortem findings; i.e. dead birds had the characteristic haemorrhagic caeca, and smears of caecal contents, stained with Giemsa, contained numerous merozoites. There is therefore the possibility of antigenic competition acting to lessen the anti-DNP response under these circumstances.

(b) The Effect of Epitope Density upon the Antibody Response in Chickens to a Hapten-Protein Conjugate

The epitope density has been shown to influence the heterogeneity of specificity and the amount of antibody produced in a mammalian species (Larralde and Janof, 1972), as well as the induction of tolerance (Feldmann, 1972b). In the present experiment therefore, the effect of the degree of hapten substitution upon anti-hapten antibody production in chickens was examined, to try and find the optimum hapten density for eliciting biosynthesis of detectable amounts of anti-DNP antibodies.

Experimental procedure Four groups of chickens (6 weeks of age) were each immunised as follows.

Group 1 : 8 mg DNP_{43}-HSA in 1 ml FCA im.

Group 2 : 10 mg DNP_{6}-HSA in 1 ml FCA im.

Group 3 : 100 µg DNP_{43}-HSA in 1 ml FCA im.

Group 4 : 100 µg DNP_{6}-HSA in 1 ml FCA im.

Epitope densities (DNP:protein molar ratios) were measured spectrophotometrically as described on page 56. Blood was taken at

TABLE 7. The effect of epitope density upon the antibody response of chickens to the carrier (HSA) following immunisation with a hapten-protein conjugate (DNP-HSA). The antibody response to the carrier moiety is expressed as the antigen-binding capacity (ABC_{30} in μg HSA ml^{-1}) for each serum sample.

BIRD NUMBER	IMMUNISATION	ABC_{30} (μg HSA ml^{-1}) AT 7 DAYS	MEAN (± SE)
909	GROUP 1 :	1.3	
910	8 mg	0.8	
911	DNP_{43}-HSA	4.2	1.9
912	in FCA im	1.2	(± 0.6)
913		3.4	
914		0.8	
134	GROUP 2 :	8.1	
135	10 mg	9.9	
136	DNP_6-HSA	16.0	9.6
137	in FCA im	11.7	(± 1.6)
138		4.1	
139		7.8	
903	GROUP 3 :	0.3	
904	100 μg	0.3	
905	DNP_{43}-HSA	0.1	0.4
906	in FCA im	0.1	(± 0.2)
907		0.3	
908		1.2	
150	GROUP 4 :	3.6	
151	100 μg	0.4	
152	DNP_6-HSA	5.7	4.9
153	in FCA im	0.2	(± 1.8)
154		8.1	
156		11.6	

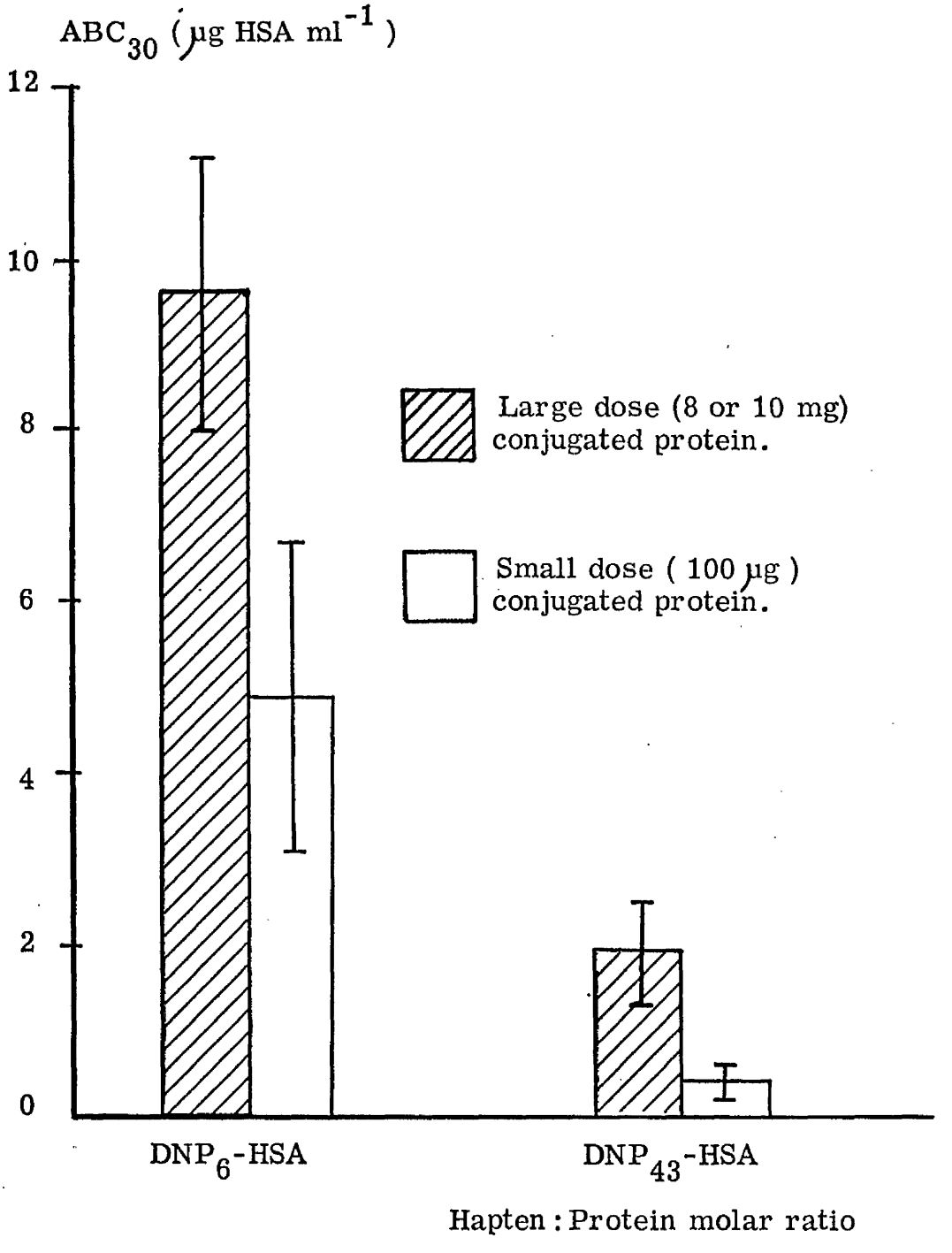


FIG. 17. The effect of epitope density upon the antibody response of chickens to the carrier (HSA) following immunisation with a DNP-HSA conjugate. The antibody response to the carrier moiety is expressed as the mean ABC_{30} (± SE) for each group of birds.

intervals after immunisation and each serum sample was assayed both for hapten-binding capacity and for carrier (HSA)-binding capacity by separate Farr tests.

Results No apparent antibody to the hapten was detected in any of the experimental groups, so that no conclusions concerning the effect of epitope density upon the antibody response to the hapten moiety could be reached. Again, this could be attributed to deficiencies in the assay system or to a genuine failure of the birds to respond to the type and amount of immunogen used.

Antibodies to the carrier (HSA) were detected however (Table 7) and at a given dose of antigen the antibody response to the carrier (i.e. the anti-HSA response) was inversely related to the epitope density (Figure 17). The simplest explanation for this is that at high epitope density the hapten groups were so numerous that they interfered sterically with the native determinants of the HSA molecule and prevented recognition of these; thus the densely substituted molecule provoked only a small anti-HSA antibody response.

(c) The Effect of Carrier Pre-Immunisation upon the Subsequent Production of Antibody in Chickens to a Hapten-Protein Conjugate

It has been shown in both mammals and birds that pre-immunisation with carrier protein alone has an enhancing effect upon the subsequent anti-hapten response when that animal is later challenged with hapten coupled to the same protein (e.g. Sarvas et al., 1974). In this experiment, the potential of carrier (HSA) pre-immunisation to enhance the subsequent production of anti-DNP antibodies by chickens was studied.

Experimental procedure Four groups of chickens (8 weeks of age) were each immunised by intravenous injection of antigens according to the following schedule.

Group 1 : Carrier pre-immunisation with 500 µg HSA iv, 7 days before immunisation with 10 mg DNP_{17}-HSA iv. (on day 0).

Group 2 : Immunisation with 10 mg DNP_{17}-HSA iv.

Group 3 : Carrier pre-immunisation with 500 µg HSA iv, 7 days before immunisation with 100 µg DNP_{17}-HSA iv. (on day 0).

Group 4 : Immunisation with 100 µg DNP_{17}-HSA iv.

The birds were bled 7 days after pre-immunisation (just before immunisation with DNP-HSA) and at invervals thereafter. Each serum sample was assayed both for hapten-binding capacity and for carrier (HSA)-binding capacity by separate Farr tests using radio-iodinated DNP-CSA and HSA respectively.

Results The results were expressed as the respective binding capacities for DNP and HSA and are given in Table 8. There was no evidence that pre-immunisation of birds with a small (500 µg) priming dose of the carrier protein caused an enhanced anti-hapten response when these birds were immunised 7 days later with DNP-HSA. Here again, only a very slight response to the DNP hapten was detected 7 days after immunisation with conjugate and the magnitude of the response was similar both in birds which were pre-immunised and in those which were not - indicating that pre-immunisation with 500 µg of the carrier had no discernible effect upon the subsequent response to a hapten when conjugated to the same protein and administered 7 days later.

TABLE 8. The effect of carrier pre-immunisation upon the biosynthesis of anti-hapten antibodies in chickens. Results are expressed as (a) anti-HSA antibody levels in terms of the antigen-binding capacity (ABC_{30} in μg HSA ml^{-1}) and (b) anti-DNP antibody levels in terms of the hapten-binding capacity (HBC_{30} in ng DNP-lysine ml^{-1}).

BIRD NUMBER	IMMUNISATION	ANTI-HSA LEVELS (ABC_{30}, μg HSA ml^{-1}) AT VARIOUS TIMES RELATIVE TO IMMUNISATION WITH DNP-HSA.			ANTI-DNP LEVELS (HBC_{30}, ng DNP-lysine ml^{-1}) 7 DAYS AFTER IMMUNISATION WITH DNP-HSA
		-30 MINS	+ 4 DAYS	+ 7 DAYS	
976	Carrier pre-	23.3	18.6	24.5	710
977	immunised;	38.1	15.8	30.2	190
978	10 mg DNP-HSA	33.3	10.1	14.3	520
979	iv. 7 days later	28.8	14.7	24.4	710
984	10 mg DNP-HSA	-	2.0	27.0	340
986	iv.	-	0.8	38.8	790
980	Carrier pre-	12.7	9.3	13.5	60
981	immunised;	20.6	14.6	6.4	-
982	100 μg DNP-HSA	22.2	20.2	16.6	30
983	iv. 7 days later	22.0	20.8	10.3	-
985	100 μg DNP-HSA	-	3.2	19.4	30
987	iv.	-	0.7	11.9	50

- not detectable

(d) The Effect of Age and Development of Chickens upon their Ability to Synthesise Anti-Hapten Antibodies

It has been suggested that certain of the immunological faculties of the chicken are not fully developed until about 5 or 6 weeks of age (Wolfe and Dilks, 1948). So far, experiments have been conducted to discover the most satisfactory conditions for elicitation of a good anti-hapten antibody response in chickens; namely by studying the advantages of different carriers and epitope densities and of carrier pre-immunisation. Finally therefore, the effect of age (and by inference, the effect of immunological maturity) upon the capability of chickens to biosynthesise anti-hapten antibodies was examined.

Experimental procedure A group of 6 adult hens (15 weeks of age) were each immunised by a single intravenous injection of 5 mg DNP_{14}-HSA in 1 ml saline; an additional bird (no. 891) was immunised by a single intravenous injection of 5 mg unsubstituted HSA. For comparative purposes, the antibody responses observed in this group of adult birds were compared with the antibody responses of the young 8-week old birds immunised with 10 mg DNP_{17}-HSA in 1 ml saline iv, which were used in the previous experiment and were of the same breed as the 6 adult birds used here. All the adult birds were bled at intervals up to 3 weeks following immunisation and the serum antibody levels of anti-DNP and anti-HSA antibodies were determined by separate Farr tests.

TABLE 9. The anti-HSA and anti-DNP antibody responses in 15-week old birds following immunisation with 5 mg DNP_{14}-HSA. Results are expressed as (a) anti-HSA antibody levels in terms of the antigen-binding capacity (ABC_{30} in µg HSA ml^{-1}) and (b) anti-DNP antibody levels in terms of the hapten-binding capacity (HBC_{30} in ng DNP-lysine ml^{-1}).

BIRD NUMBER	IMMUNISATION	DAYS AFTER IMMUNISATION 3	5	7	9	12	14	21
		SERUM ANTI-HSA ANTIBODY LEVELS (ABC_{30}, µg HSA ml^{-1})						
882	5 mg DNP-HSA iv.	<0.1	24.4	18.4	7.8	2.0	0.7	0.3
883	" "	<0.1	21.1	10.3	5.9	1.2	0.4	0.2
884	" "	<0.1	53.1	53.9	24.4	8.9	3.9	1.9
885	" "	<0.1	27.2	13.3	6.4	2.9	1.4	0.5
886	" "	<0.1	63.3	66.6	39.0	17.1	8.1	1.8
887	" "	<0.1	27.7	18.8	6.3	1.7	0.5	0.1
891	5 mg HSA iv.	nd	7.5	58.8	53.6	nd	nd	5.8

BIRD NUMBER	IMMUNISATION	DAYS AFTER IMMUNISATION 3	5	7	9	12	14	21
		SERUM ANTI-DNP ANTIBODY LEVELS (HBC_{30}, ng DNP-lys ml^{-1}						
882	5 mg DNP-HSA iv.	42.6	957.2	1313.6	436.7	430.2	468.2	139.0
883	" "	48.7	493.1	810.0	331.6	158.6	317.8	163.2
884	" "	33.9	866.8	1512.5	1426.6	1091.9	492.2	215.4
885	" "	45.4	631.2	595.3	471.1	398.3	255.5	100.8
886	" "	53.8	581.0	782.8	1110.1	574.6	375.2	157.1
887	" "	33.7	666.3	441.1	1013.5	657.9	315.8	93.8
891	5 mg HSA iv.	nd	28.3	26.6	17.3	nd	nd	-

\- not detectable
nd not determined

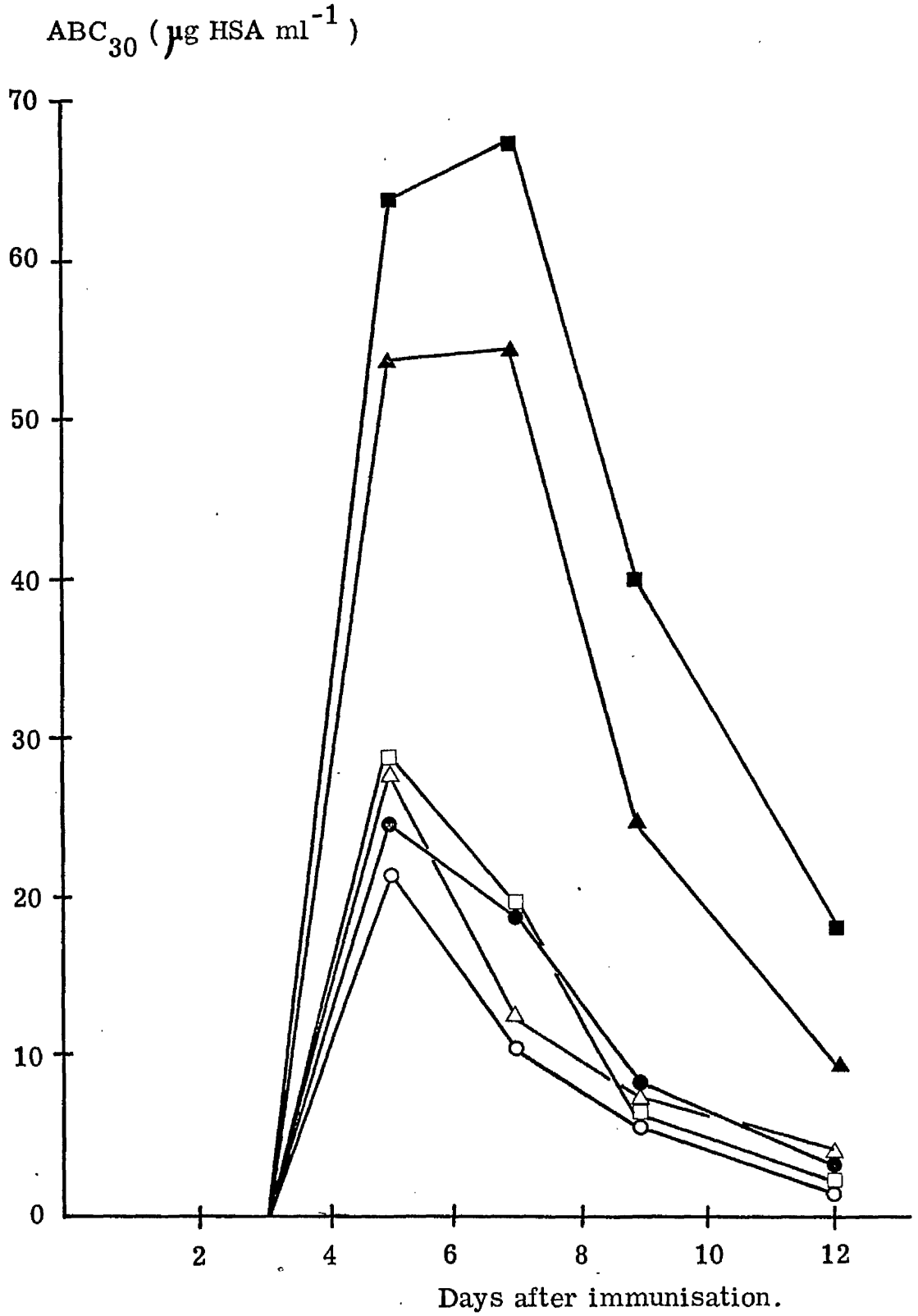


FIG. 18. The anti-HSA antibody response in a group of six 15-week old birds following immunisation with 5 mg DNP_{14}-HSA.

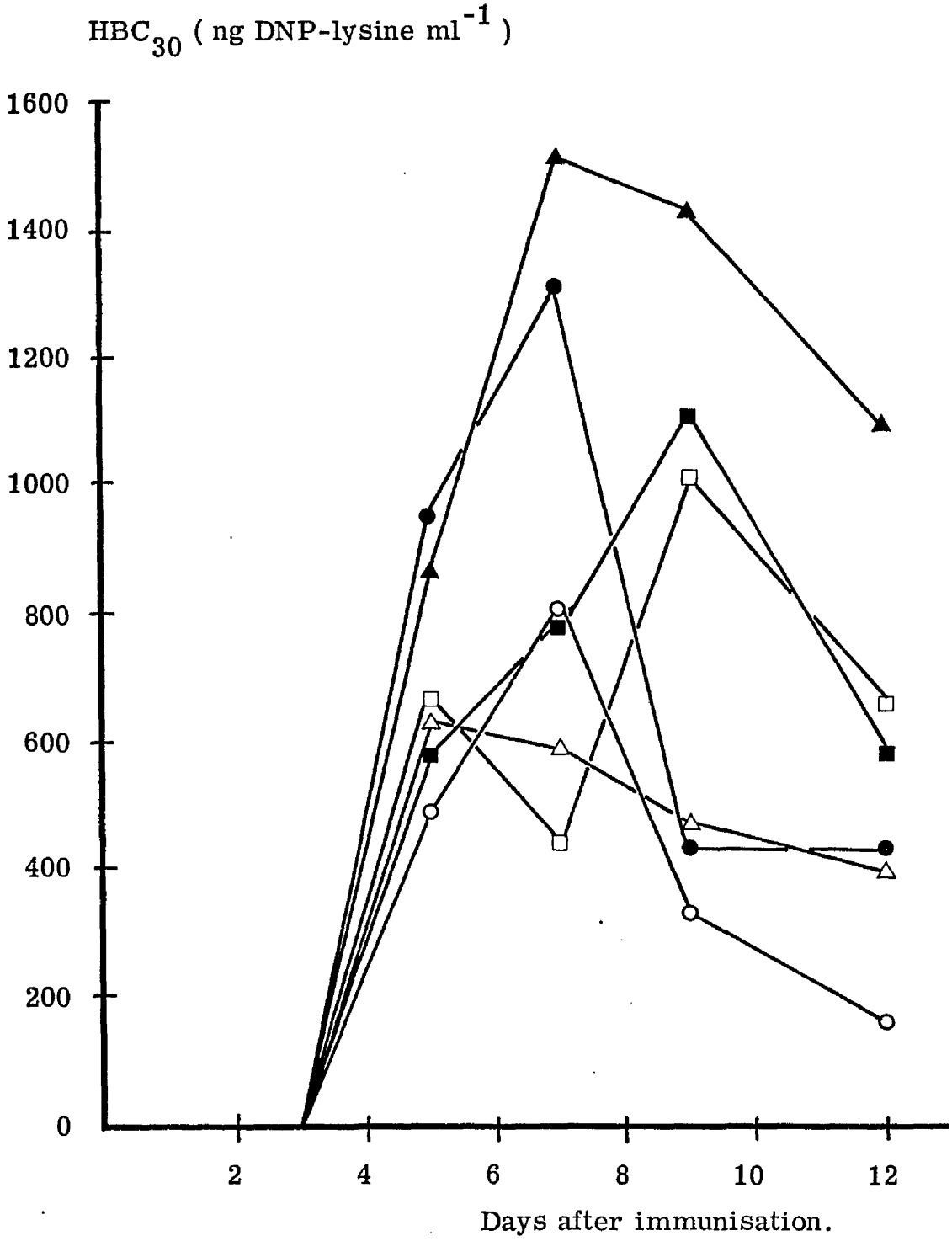


FIG. 19. The anti-DNP antibody response in a group of six 15-week old birds following immunisation with 5 mg DNP_{14}-HSA.

Results The results were expressed as the binding capacities of each serum sample for HSA and DNP; these values are given in Table 9 and the anti-HSA and anti-DNP responses are shown respectively in Figures 18 and 19. Just as with the young 8-week old birds used in the previous experiment (3c) (Table 8), adult birds readily produced antibodies specific for the HSA carrier. However, the older birds were much more capable of synthesising detectable amounts of anti-DNP antibodies (Figure 19) than the 8-week old birds. Thus, despite previous unsuccessful attempts to elicit good anti-DNP responses in chickens, a quantitative response was observed in adult birds using the immunisation regimen described. The specificity of anti-DNP biosynthesis and of the assay system used for its detection was confirmed by the failure to demonstrate DNP-binding activity in serum samples from bird no. 891 - which received only unsubstituted HSA (Table 9). These results therefore suggest that birds develop the capacity to respond to haptens as they become older; in this instance, the critical age was apparently greater than 8 weeks and less than 15 weeks.

Experiment 4. The Effect of Immunising Chickens with a Hapten-Carrier Conjugate (DNP-HSA) and Challenging Either with Carrier Alone (HSA) or with the Hapten on a Different Carrier

The results of the previous experiment implied that the age of the birds was an important consideration in successfully immunising chickens to the DNP hapten. So as not to lose sight of one of the original objectives (i.e. to elicit biosynthesis of detectable

amounts of reasonably high affinity antibody and to discover how different immunisation procedures affected this), the facility of adult birds for secondary stimulation was studied.

Experimental procedure The same group of 6 adult hens used in the previous experiment (Experiment 3d; immunisation with 5 mg DNP_{14}-HSA iv.) was divided into two groups of 3, and each group was challenged, 40 days after priming, as follows:

Group 1 : secondary immunisation with 5 mg HSA in 1 ml saline iv.
Group 2 : secondary immunisation with 2 mg DNP-HCN in 1 ml saline iv.

The birds were bled at intervals following secondary immunisation, and serum samples were assayed for both DNP- and HSA-binding activity.

Results The results were expressed as the binding capacities of each serum sample for HSA and DNP; these values are given in Table 10 and the anti-DNP and anti-HSA responses are shown respectively in Figures 20 and 21.

In birds in Group 1 which were primed with DNP-HSA and re-immunised with unsubstituted HSA, the secondary antibody response to the carrier was greater than in the primary response (Table 10; Figure 21) but no anti-hapten activity was found. Chickens in Group 2, which had been primed with DNP-HSA and then re-immunised with DNP conjugated to a different carrier (haemocyanin), showed a rapid (i.e. detectable after 3 days) secondary antibody response to the hapten (Table 10; Figure 20) - but no antibody to the original carrier, HSA, was found.

TABLE 10. The effect of immunising chickens with a hapten-carrier conjugate (DNP-HSA) and challenging 40 days later either with carrier alone (HSA) or with the hapten on a different carrier (DNP-HCN). Results are expressed as (a) anti-HSA antibody levels in terms of the antigen-binding capacity (ABC_{30}, µg HSA ml^{-1}) and (b) anti-DNP antibody levels in terms of the hapten-binding capacity (HBC_{30}, ng DNP-lys ml^{-1}).

BIRD NUMBER	PRIMARY IMMUNISATION	DAYS AFTER 1°			SECONDARY IMMUNISATION	DAYS AFTER 2°					
		5	7	12		2	3	5	7	10	14
		ANTI-HSA LEVELS				ANTI-HSA LEVELS					
882	GROUP 1 :	24.4	18.4	2.0	GROUP 1 :	<0.1	0.4	54.8	71.1	42.3	15.3
883	5 mg DNP-	21.1	10.3	1.2	5 mg HSA iv.	0.2	2.7	41.1	33.9	13.4	12.0
884	HSA iv.	53.1	53.9	8.9		<0.1	0.5	103.4	184.5	134.9	63.0
885	GROUP 2 :	27.2	13.3	2.9	GROUP 2 :	0.2	0.4	0.9	0.4	2.1	nd
886	5 mg DNP-	63.3	66.6	17.1	2 mg DNP-HCN	2.4	3.1	2.8	2.4	3.9	nd
887	HSA iv.	27.7	18.8	1.7	iv.	0.1	0.2	0.3	0.6	0.3	nd

BIRD NUMBER	PRIMARY IMMUNISATION	DAYS AFTER 1°			SECONDARY IMMUNISATION	DAYS AFTER 2°					
		5	7	12		2	3	5	7	10	14
		ANTI-DNP LEVELS				ANTI-DNP LEVELS					
882	GROUP 1 :	957.2	1313.6	430.2	GROUP 1	53.0	112.5	70.6	103.1	76.1	nd
883	5 mg DNP-	493.1	810.0	158.6	5 mg HSA iv.	93.0	72.3	114.4	84.7	30.1	nd
884	HSA iv.	866.8	1512.5	1091.9		96.3	100.9	231.9	183.6	108.5	nd
885	GROUP 2 :	631.2	595.3	398.3	GROUP 2 :	102.4	1814.8	5665.6	3951.9	1620.5	1106.5
886	5 mg DNP-	581.0	782.8	574.6	2 mg DNP-HCN	152.2	1932.9	6811.4	5059.3	2915.4	1790.3
887	HSA iv.	666.3	441.1	657.9	iv.	72.0	1176.4	6234.7	3095.3	1859.7	937.1

nd not determined

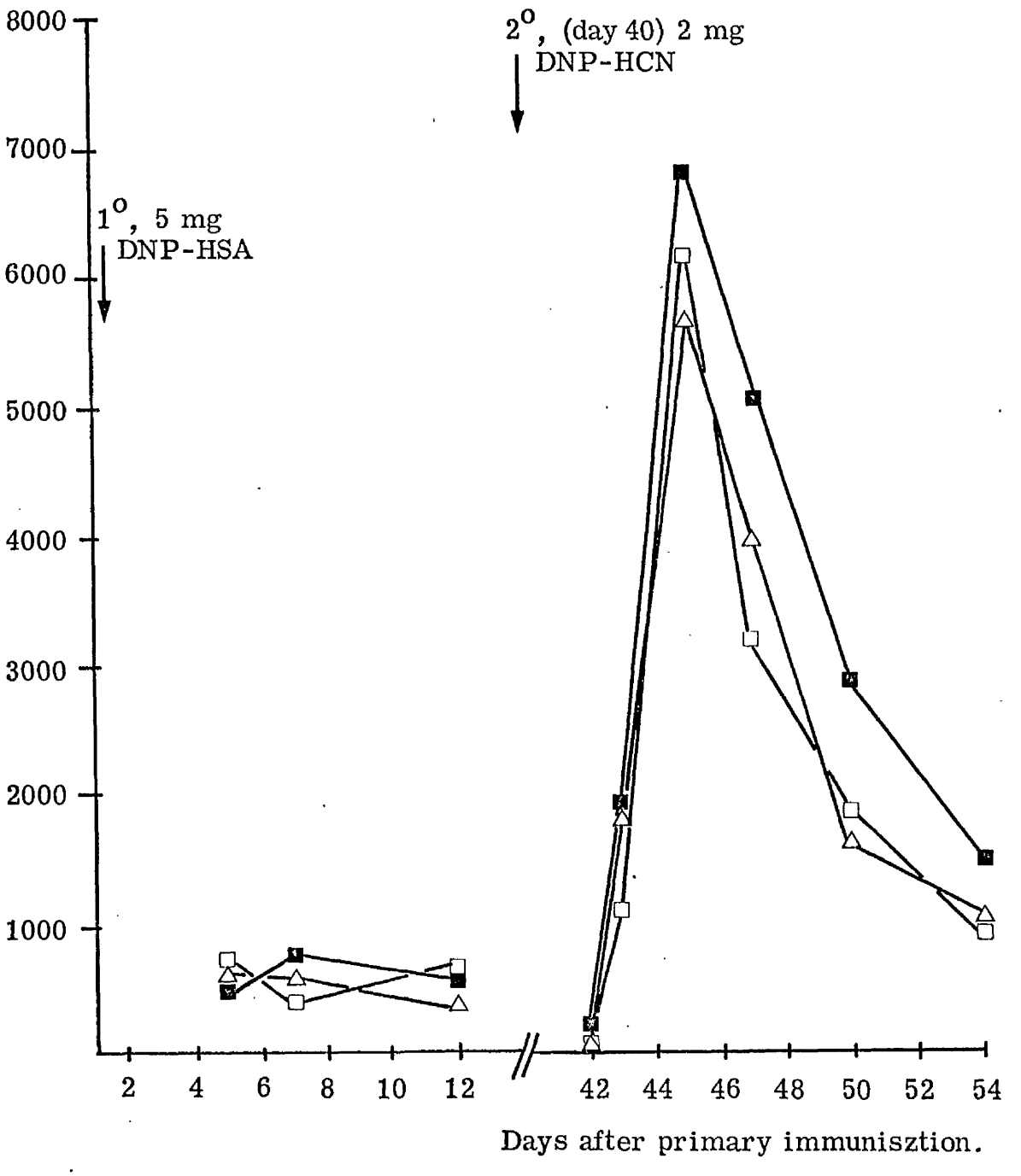


FIG. 20. The effect of immunising chickens with a hapten-carrier conjugate (DNP-HSA) and challenging 40 days later with the same hapten on a different carrier (i.e. DNP-HCN) upon the anti-DNP antibody response.

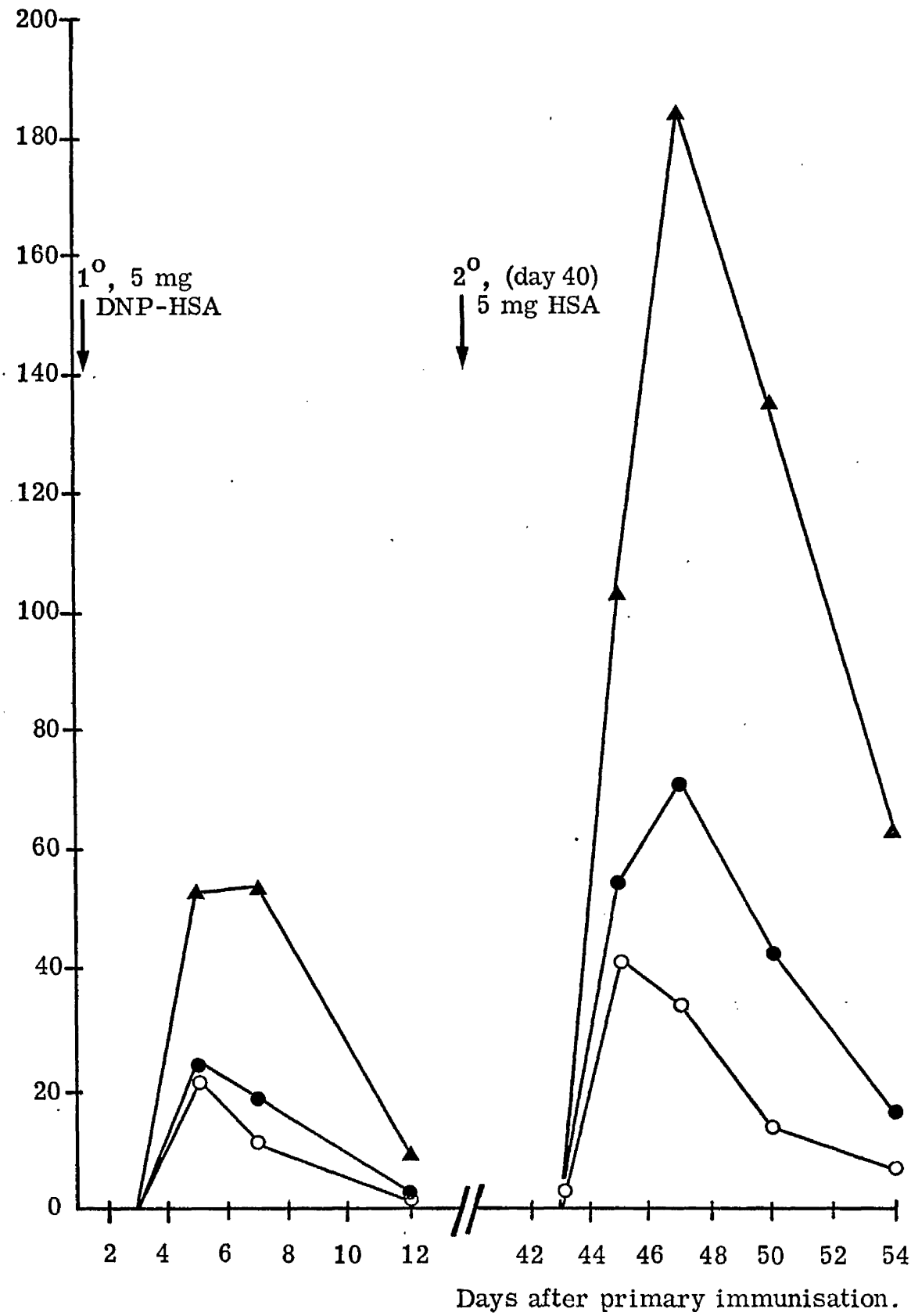


FIG. 21. The effect of immunising chickens with a hapten-carrier conjugate (DNP-HSA) and challenging 40 days later with the carrier alone upon the anti-HSA antibody response.

Secondary immunisation therefore caused production of antibodies specific only for those antigenic determinants used in the secondary stimulus. Indeed, the DNP hapten presented a second time on a heterologous carrier, caused very rapid anti-DNP biosynthesis and increased amounts of antibody were produced; the kinetics of the response (e.g. rapid onset of biosynthesis) and its magnitude suggested that the response was anamnestic - in which case, there was no apparent requirement of the immunological memory for carrier specificity. Alternatively, the pattern of the so-called secondary response reflected the greater efficiency of haemocyanin as a carrier compared with HSA, in a situation where no memory had been imparted.

When the levels of anti-HSA antibody in the serum at the 7-day peak of the primary antibody response of birds in Group 1 were compared with the secondary responses at day 47, a close correlation between them was evident (i.e. correlation coefficient = 0.998; the probability of obtaining so high a value by chance was $P = 0.02$). The primary and secondary anti-DNP levels of birds in Group 2 were too similar to each other at 7 days and at 47 days to allow any meaningful comparison to be made.

II. The Affinity of Anti-Hapten and the Avidity of Anti-Protein Chicken Antibodies

Experiment 5. The Effect of Antigen Dose upon the Affinity of Chicken Antibodies for DNP-EACA

If it is assumed that a process of cell selection does

take place and involves the interaction of antibody-like receptors with antigen, then it would be expected that the affinity of serum antibody for antigen would depend on the valency and dose of the antigen to which the individual is responding. In mammalian species, the average affinity of antibodies has generally been shown to correlate with the dose and type (e.g. structure and valency) of the antigen, as well as with the method of immunisation and the duration of a response.

In this experiment an attempt was made to relate the average affinity of chicken anti-hapten antibodies to the dose of immunogen.

Experimental procedure Two groups of chickens (10 weeks of age) were each immunised as follows.

Group 1 : 10 mg DNP_{24}-HSA in 1 ml saline iv.

Group 2 : 100 µg DNP_{24}-HSA in 1 ml saline iv.

Blood samples (20 ml) were taken by cardiac puncture from each bird 7 days after primary immunisation. A globulin fraction was prepared from each serum sample, and these globulin fractions were assayed by equilibrium dialysis for their affinity for the ligand DNP-EACA.

The values for free and bound hapten concentrations, [c] and [b], were obtained from the equilibrium dialysis data (as described on page 100). Next, a plot of $\log_{10}$ [b] against $\log_{10}$ [c] was constructed for each serum sample, and from this graph the values for the heterogeneity index and for the mean intrinsic association

TABLE 11. The effect of antigen dose upon the affinity, amount and heterogeneity of anti-DNP antibodies produced by chickens 7 days after immunisation with DNP-HSA. Results are expressed as (a) the affinity, K_o (litres mole^{-1}) (b) the hapten-binding capacity, HBC_{30} (ng DNP-lys ml^{-1}) and (c) the index of heterogeneity, a.

BIRD NUMBER	IMMUNISATION	7 DAYS AFTER IMMUNISATION		
		K_o (litres mole^{-1})x 10^3	HBC_{30} (ng DNP-lys ml^{-1})	INDEX OF HETEROGENEITY (a)
980	GROUP 1 :	7.8	82.6	1.0
981	10 mg DNP-HSA	0.2	73.9	1.0
982	iv.	7.8	467.9	1.0
988		6.3	168.0	1.0
989		9.8	334.2	1.0
990		4.9	203.5	1.0
	MEAN K_o (±SE) 6.1 (±1.4)			
984	GROUP 2 :	3.4	72.4	1.0
985	100 µg DNP-HSA	0.2	117.6	1.0
987	iv.	0.6	271.3	1.0
	MEAN K_o (±SE) 1.4 (±1.0)			

constant were derived (as described on pages 100 and 103).

Results The results were expressed in terms of the geometric mean intrinsic association constant (K_o, litres $mole^{-1}$), the heterogeneity index (a) and the hapten-binding capacity of the serum (HBC_{30}, ng DNP-lys ml^{-1}); these values are given in Table 11. Average affinities of serum antibodies following immunisation with either 10 mg or 100 µg of DNP-HSA were very low, in the range $K_o = 10^2$ to 10^4 litres $mole^{-1}$. The mean value for the affinity at 7 days in the group immunised with 10 mg of DNP-HSA was unexpectedly higher than that in the group immunised with 100 µg DNP-HSA; this observation was contrary to the prediction based on a cell selection theory, that lesser amounts of antigen would have stimulated only those clonotypes of high affinity. However, on statistical testing it appeared that the difference between the two groups might conceivably have arisen by chance - the probability of this happening (as judged by the two-tailed t-test) gave P = 0.059.

In both experimental groups the index of heterogeneity was invariably 1.0, which implied that there was an homogeneous population of antibodies. There was no correlation between the absolute amount and the affinity of serum antibodies.

One important question which is posed by these results concerns the very low affinities which were obtained. If the receptor sites on the lymphocytes had the same low affinity for hapten as antibodies in the serum had, then it is difficult to see how any number of lymphocytes could have been stimulated at all.

Experiment 6. The Effect of Repeated Immunisation upon the Amount and the Avidity of Anti-HSA Antibodies Produced by Chickens

The results of the previous experiment indicated that immunisation of chickens with a hapten-protein conjugate evoked the biosynthesis of antibodies of very low average affinity for the hapten. The simplest explanation for this is that the birds were incapable of biosynthesising detectable amounts of medium or high affinity anti-DNP antibodies when immunised with the antigen at the doses and according to the procedures employed in the previous experiment.

An alternative approach to studying the effects of cell selection upon the quality of antibody produced was therefore tried; this was to measure the avidity of chicken antibodies for protein antigens, and in particular in the situation where antigen was repeatedly administered to the bird.

Experimental procedure Four chickens (12 weeks of age) were each immunised by an initial intravenous injection of 100 µg HSA (in 1 ml saline). Thereafter each bird was given repeated doses of 100 µg HSA at weekly intervals for 4 weeks. Each week the birds were bled just before injection of HSA, as well as on the day preceding and on the day following injection. The values for the avidity, K_R, and the total antigen-binding capacity, $[Ab_t]$, were derived from a least squares linear plot of 1/[b] (reciprocal of the bound HSA concentration) against 1/[c] (reciprocal of the free HSA concentration) - using both the IBM 370/155 computer and a Hewlett Packard 9100 B calculator, as described on pages 89 and 91.

Results The results were expressed in terms of K_R and $[Ab_t]$, and these values are given in Table 12. The values for avidity showed little variation during the initial three weeks following the first injection of antigen, but there was a significant increase in avidity during the fourth week (i.e. the mean value of K_R for day 20 tested against the mean value of K_R for day 27 gave $P = 0.017$ by the two-tailed t-test; likewise the mean value of K_R for day 21 tested in the same way against the mean value of K_R for day 28 gave $P = 0.034$). This pattern of a significant increase in the mean avidity occurring during the fourth week after the first injection of antigen was reflected in the pattern of antigen-binding capacities, which also rose markedly during the fourth week. Some increase in the antigen-binding capacity did however occur during the third week, and it was interesting that this presaged the increase in avidity (which was only detected at the end of the fourth week) : on the basis of a cell selection theory, a plausible explanation would be that the greater amounts of antibody present in the serum during the third week were appropriating more of the injected antigen, and therefore only cells bearing high avidity receptors were being triggered to respond in the fourth week.

The antigen-binding capacities at the end of the second week after the first injection of antigen (i.e. after 13, 14 and 15 days) were lower in all experimental birds when compared with the serum antigen-binding capacity in the same individual 7 days previously (i.e. at the end of the first week). This refractory phase suggested that efficient homeostasis was in operation, preventing further stimulation of antibody biosynthesis following the second

TABLE 12. The effect of repeated immunisation at seven-day intervals upon the avidity and the amount of anti-HSA antibodies produced by chickens. Results are expressed as (a) the avidity, K_R (litres $mole^{-1}$) and (b) the total antigen-binding capacity.

BIRD NUMBER	IMMUNISATION	DAYS AFTER PRIMARY IMMUNISATION											
		6	7	8	13	14	15	20	21	22	27	28	29
		ANTI-HSA AVIDITY, K_R (litres $mole^{-1}$) x 10^{-5}											
65	Initial dose of	3.2	3.4	3.1	6.2	4.4	3.4	3.9	4.3	4.3	7.4	6.6	6.3
66	100 µg HSA iv;	5.5	6.1	4.4	4.8	6.5	2.4	5.0	4.4	5.3	10.3	8.0	5.4
67	further 100 µg	4.3	4.6	5.0	3.5	3.4	2.9	3.1	3.0	4.2	5.8	4.9	4.4
68	HSA injected iv. every 7th day for 4 weeks	3.8	3.9	4.3	3.4	3.6	4.3	5.5	5.3	5.9	10.4	9.6	8.4

BIRD NUMBER	IMMUNISATION	TOTAL ANTIGEN-BINDING CAPACITY, $[Ab_t]$ (µg HSA ml^{-1})											
65	As above.	40.0	32.7	19.0	22.6	19.0	12.8	88.7	72.4	63.5	206.0	205.4	178.9
66		66.4	64.7	47.1	37.3	27.9	25.1	98.2	88.1	55.5	237.2	210.5	175.6
67		73.2	103.9	99.6	67.1	62.3	56.0	74.9	61.1	39.9	196.2	171.0	148.4
68		85.5	97.6	38.8	75.8	67.8	46.3	152.5	138.2	114.3	230.1	222.4	197.4

injection of antigen. However, this homeostatic effect was overcome during the third and fourth weeks, as evidenced by the significant increases in the antigen-binding capacity found in serum samples from each bird at this time.

EVIDENCE FOR ACCESSORY CONTROL MECHANISMS

The experimental work described so far has been concerned with the effect of structure and amount(s) of antigen(s) injected upon those parameters (i.e. the antigen-binding capacity, affinity, avidity and heterogeneity) which have been taken as markers of antibody biosynthesis and thus of cell selection. However, there are other regulatory factors which are capable of modifying the course of antibody biosynthesis - either at cell selection or at a later stage. For example, the use of adjuvants to enhance a response, and the immunosuppression achieved by passive antibody (and the naturally occurring equivalent of this, namely negative feedback inhibition) can be counted as regulators of antibody biosynthesis, although the level at which they operate is a matter for discussion.

I. The Effects of Adjuvants upon Antibody Biosynthesis in Chickens

Experiment 7. The Effect of Freund's Complete Adjuvant upon the Kinetics of Anti-HSA Antibody Biosynthesis in Chickens

The inclusion of antigen in a water-in-oil emulsion which also contains mycobacteria and which is injected into the breast muscle of a chicken might be expected to modify the antibody

response to that antigen as compared to the response observed following immunisation by straightforward intravenous injection of antigen in solution; such modification might be expected for two reasons at least. First of all, the antigen solution is restricted to the dispersed aqueous phase of the water-in-oil emulsion which is injected into the muscle of the bird; in view of this it would be predicted that removal of antigen from the site of injection in the tissue and its encounter with the cells of the immune system is a protracted albeit continuous process, although the actual kinetics of antigen removal have not been confirmed by experiment. The second point to consider is the presence of mycobacteria in the adjuvant mixture; mycobacteria are classically associated with the chronic granulomatous type of cell reaction, in which circulating cells migrate from the bloodstream and accumulate to form a solid granuloma. The development of lymphoid nodules in the later stages of granuloma formation (White, Coons and Connolly, 1955) and also the extraction of considerable amounts of specific antibody from adjuvant granuloma tissue in chickens (French, Stark and White, 1970) suggest that, in the chicken at least, the granuloma which develops following immunisation with antigen in FCA is the site of at least some, if not most, of the production of specific antibody.

The present experiment was carried out to examine the effects of FCA upon the kinetics of antibody biosynthesis following immunisation with either 5 mg or 100 µg HSA in FCA. The intention was to compare the kinetics of this response (including the relationship between antibody levels in the serum and in the granuloma tissue

of each bird) with the pattern of antibody production following immunisation of chickens with antigen in saline in the absence of adjuvant, i.e. to study how the gradual release of antigen together with the inclusion of mycobacteria in the adjuvant influenced the kinetics of biosynthesis; from this it was hoped to form some conclusions as to how these two properties of the adjuvant were effective at the cellular level.

Experimental procedure Two groups of birds (8 weeks of age) were used; the first group of 6 birds were each immunised by a single intramuscular injection of 5 mg HSA in FCA and the second group of 6 birds were each immunised by a single intramuscular injection of 100 µg HSA in FCA. In each case, 0.5 ml of adjuvant (containing 5 mg M. avium in the oil phase and with the antigen included in the aqueous phase) was injected into the left breast muscle. The birds were bled at regular intervals up until 7 weeks following immunisation and serum anti-HSA antibody levels were determined by the Farr test. After the final bleeding at 48 days the birds were killed to enable quantitation of the antibody in the granuloma which had developed at the site of injection in individual birds.

Results The results were expressed as the antigen-binding capacity of each serum sample at various times after immunisation, (and of the antibody-containing extract prepared from the granuloma tissue of each bird 48 days after immunisation) and these values are given in Table 13, whilst mean values of serum antibody levels at the two doses of antigen used are shown in Figure 22.

TABLE 13. The effect of Freund's complete adjuvant upon the kinetics of anti-HSA antibody biosynthesis in chickens. Results for serum antibody levels are expressed in terms of the antigen-binding capacity, ABC_{30} (µg HSA ml^{-1}) and similarly granuloma extract antibody levels are expressed as ABC_{30} (µg HSA per 100 mg tissue).

BIRD NUMBER	IMMUNISATION	SERUM ANTIBODY LEVELS (ABC_{30}, µg HSA ml^{-1}) AFTER 7	12	24	36	48 DAYS	GRANULOMA ANTIBODY (ABC_{30}, µg HSA/100 mg) AFTER 48 DAYS
134	5 mg HSA in	8.1	2.2	0.7	2.9	13.4	6.5
135	FCA im.	9.9	2.4	*	*	*	*
136		16.0	7.7	1.7	9.6	20.5	10.9
137		11.7	5.6	2.2	8.3	17.9	7.4
138		4.1	2.1	0.8	2.4	2.6	0.3
139		7.8	3.4	2.3	20.4	36.2	9.2
MEAN (±SE)		9.6(±1.6)	3.9(±0.9)	1.5(±0.3)	8.7(±3.2)	18.1(±5.5)	6.9(±1.8)
150	100 µg HSA	3.6	4.3	2.4	5.4	5.1	1.5
151	in FCA im.	0.4	10.3	2.6	0.4	*	*
152		5.7	5.8	4.3	2.0	1.0	0.6
153		0.2	2.4	0.3	0.1	0.4	nd
154		8.1	16.9	34.0	36.7	34.3	5.3
156		11.6	6.4	2.4	12.9	20.2	4.0
MEAN (±SE)		4.9(±1.8)	7.7(±2.1)	7.7(±5.3)	9.6(±5.7)	12.2(±6.6)	2.9(±1.1)

* bird died
nd not determined

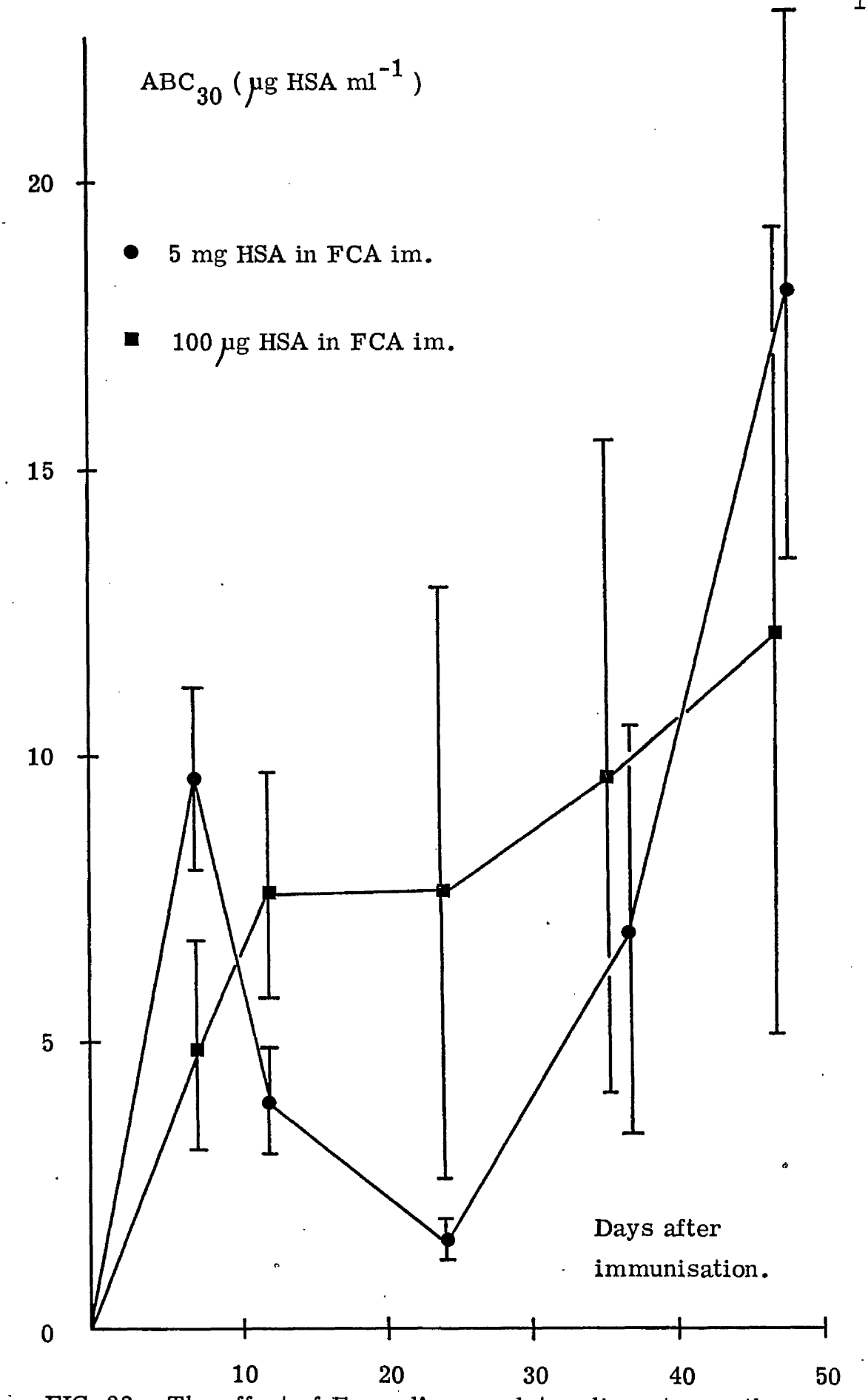


FIG. 22. The effect of Freund's complete adjuvant upon the kinetics of anti-HSA antibody biosynthesis in chickens.

The kinetics of antibody biosynthesis following immunisation with either 5 mg or 100 µg of HSA in FCA indicated that in the majority of birds (i.e. 4/5 and 3/6 respectively for the two antigen concentrations) there was a biphasic antibody response. These birds showed an initial phase of biosynthesis, the kinetics of this phase being very similar to the pattern observed following intravenous administration of antigen in saline (e.g. as shown in Figure 15, page 119); thus, once peak levels of antibody had been attained after about 7 days there was at least some decline observed in those birds which had been immunised with 5 mg HSA, whereas chickens which had been given 100 µg HSA showed a levelling off rather than any diminution of antibody levels (Figure 22). It was noticeable that the few birds immunised with HSA in FCA and which failed to show a definite biphasic pattern of antibody production, were coincidentally the same birds which showed a poor response at 7 days after immunisation. However, a second phase of antibody biosynthesis was observed in most of the experimental birds, and this was first detected between 3 and 4 weeks after immunisation. The levels of antibody in the serum rose steadily and there was no indication of a decline before 48 days. This pattern of antibody biosynthesis is in agreement with that described by French, Stark and White (1970) who reported a similar biphasic response in chickens immunised with 40 µg HSA in FCA.

These results would suggest that either the feedback homeostasis which normally operates from about 7 days after immunisation becomes ineffective with the passage of time and therefore a second phase of antibody biosynthesis is stimulated by the antigen which is

still present at the site of injection, or else that homeostasis has been actively alleviated, for example by the disruptive action of mycobacteria upon germinal centres containing segregated B cells (the mechanism proposed by White, 1973), again giving the chance for antigen still in the tissues to provoke a second phase of antibody biosynthesis. In both cases the presence of mycobacteria might well cause potentiation of the secondary response.

The kinetics of the second phase of the antibody response observed in birds following immunisation with antigen in complete adjuvant were dissimilar to the pattern seen after a second intravenous injection of antigen in solution (e.g. 40 days after a first injection), as shown in Figure 21 on page 141. In a genuine secondary response there was good evidence that feedback homeostasis was just as effective (i.e. a rapid decline in serum antibody levels was observed once the peak had been reached 5 to 7 days after secondary injection) as in the primary response. This rapid decline, as evidence of efficient homeostasis, was not seen in the adjuvant-assisted antibody response; in fact the duration of the second phase of biosynthesis, which began between 20 and 30 days after immunisation and showed no signs of diminishing by 48 days, suggested that homeostasis was not in operation and this, with the slow release of antigen from the tissues at the site of injection, was contributing towards such a protracted second phase.

There was a significant correlation of antigen-binding capacity of serum with antigen-binding capacity of granuloma extract when samples obtained 48 days after immunisation of chickens with HSA

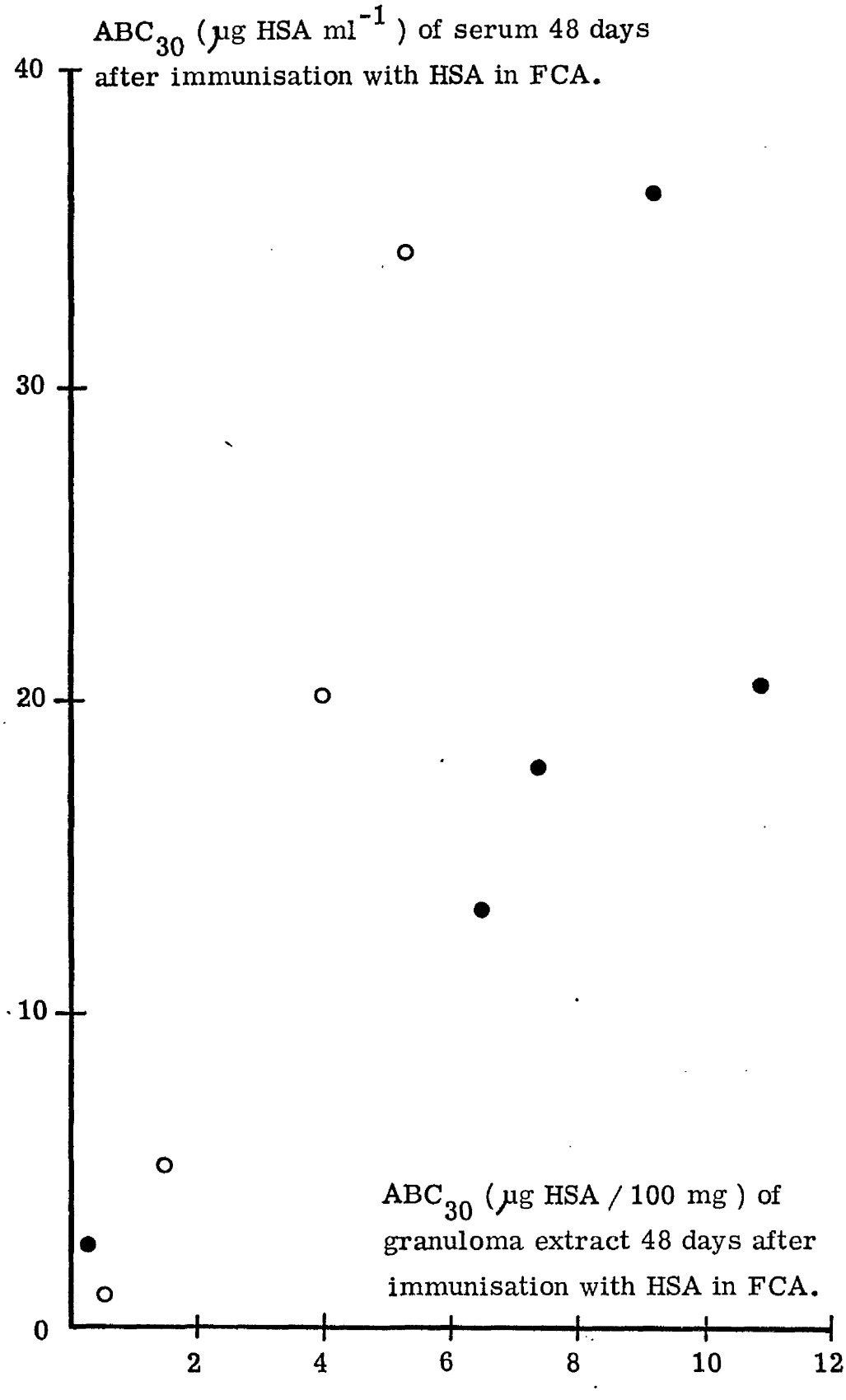


FIG. 23. Correlation of antigen-binding capacity of serum with antigen-binding capacity of granuloma extract for samples obtained 48 days after immunisation of chickens with either 5 mg (●) or 100 μg (○) HSA in FCA.

in FCA were compared (see Figure 23); for birds immunised with 5 mg HSA the correlation coefficient was 0.788 and for chickens immunised with 100 µg the correlation coefficient was 0.989 (the significance probability values of these two correlations being $P = 0.057$ and $P = 0.0057$ respectively). The most likely explanation for these significant correlations is that most of the antibody biosynthesis in the second phase of the adjuvant-assisted response was taking place within the granuloma rather than in the spleen or elsewhere, which would concur with the findings of French, Stark and White (1970). The fact that the correlation between the antigen-binding capacity in the serum and that in the granuloma extract was not as good for chickens immunised with 5 mg HSA in FCA as for those birds given 100 µg HSA in FCA could be attributed to the relatively large amounts of antigen still present in the tissues of birds in the first group combining with the antibody produced within the granuloma, effectively lowering the antigen-binding capacity of the tissue and increasing the error in its measurement.

Experiment 8. The Effects of Freund's Complete and Incomplete Adjuvants upon the Avidity of Anti-HSA Antibodies in Chickens

The results of the previous experiment showed that the anti-HSA response in chickens immunised with antigen in FCA, had two distinct phases of biosynthesis. In order to explain this phenomenon White (1973) has suggested that the adjuvant actively causes feedback homeostasis to be relaxed, and that antigen still present in the adjuvant depot in the muscle then elicits a second antibody response. Another possibility is that feedback is ablated spontaneously, and

that residual antigen then provokes a subsequent response - potentiated perhaps by the inclusion of mycobacteria. A third alternative is that the later phase of antibody biosynthesis depends on a population of, say, T cells, which take a long time to proliferate or on an entirely different population of B cells. In any case, it would appear that adjuvant regulates the response at some stage subsequent to the initial selection and triggering of antibody-producing cells, as well as affecting these processes directly.

The quality of antibody might be expected to reflect the way in which adjuvant affects an established antibody response; if it is supposed that a similar spectrum of antibody-producing cell clones which were active in the first phase is restimulated in the second phase, then overall increases in avidity between first and second phases (increases determined by the passage of time and by the exponentially diminishing amounts of antigen released by the adjuvant depot) might be predicted. On the other hand, if a different population of uninitiated antibody-producing cells is involved in the second phase of antibody biosynthesis, then an equal or lower avidity in the second phase would be anticipated.

The present experiment was carried out to study the avidity of antibody from the first and second phases of the adjuvant-mediated antibody responses, and to compare these values with the avidities of antibody obtained from a primary antibody response in chickens immunised with antigen without adjuvant.

Experimental procedure Three groups of birds (12 weeks of age) were each immunised by a single intramuscular injection of 40 µg of

HSA as follows.

Group 1 : 40 µg HSA in FCA, 0.5 ml, im.

Group 2 : 40 µg HSA in FIA, 0.5 ml, im.

Group 3 : 40 µg HSA in saline, 0.5 ml, im.

The birds were bled at intervals following immunisation and antibody avidities were quantitated.

Results The results were expressed as the relative association constant (K_R) for each serum sample, and these values are given in Table 14. Between 5 and 12 days after immunisation with HSA in FCA there was a significant increase ($P = 0.012$) in avidity - which was reconcilable with a process of cell selection whereby avidity would be expected to increase when the antigen concentration decreased as a function of time after immunisation. Thereafter the avidity appeared to decline, but this decline was of very doubtful statistical significance ($P = 0.075$ by two-tailed t-test).

Birds in Group 1 (immunised with HSA in FCA) showed a mean avidity after 12 days which appeared greater than the corresponding mean values for birds in both Groups 2 and 3, but this was not confirmed by subsequent statistical testing ($P = 0.22$ and $P = 0.06$ respectively). As well as this, the overall changes in avidity for birds in each of Groups 1, 2 and 3 between 5 and 29 days (i.e. between the first and second phases of antibody biosynthesis for chickens in Groups 1 and 2 immunised with HSA in FCA and FIA) were not significant ($P > 0.05$), neither were the changes observed between 5 and 12, and between 12 and 29 days significant for birds in Groups 2 and 3.

TABLE 14. The effects of Freund's complete and incomplete adjuvants upon the avidity of anti-HSA antibodies in chickens. Results are expressed as the avidity, K_R (litres $mole^{-1}$), for individual serum samples.

BIRD NUMBER	IMMUNISATION	DAYS AFTER IMMUNISATION		
		5	12	29
		AVIDITY, K_R (litres $mole^{-1}$) x 10^{-5}		
250	GROUP 1 :	3.9	11.2	11.5
251	40 µg HSA	4.3	12.6	4.4
252	in FCA im.	2.9	21.7	4.6
253		4.0	9.2	6.5
	MEAN (±SE)	3.8(±0.3)	13.68(±2.76)	6.75(±1.65)
254	GROUP 2 :	1.9	15.8	15.0
255	40 µg HSA	1.3	1.5	2.2
257	in FIA im.	4.3	2.6	5.0
	MEAN (±SE)	2.50(±1.92)	6.63(±4.59)	7.40(±3.89)
258	GROUP 3 :	7.3	nd	1.5
259	40 µg HSA	0.4	1.5	nd
260	in saline im.	2.0	4.3	5.9
261		11.1	8.5	nd
	MEAN (±SE)	5.20(±2.46)	4.77(±2.03)	3.70(±2.20)

nd not determined

Experiment 9. The Effect of Freund's Complete Adjuvant upon the Relative Amounts of 19S and 7S anti-HSA Antibodies in Chickens. Comparison of this Proportion with that Found in Primary and Secondary Antisera from Birds Immunised Without Adjuvant.

The previous experiment showed that there was no significant change in avidity between the early (5 or 12 day) and late (29 day) phases of the adjuvant-assisted antibody response in chickens; this in turn suggested that there was no shift in the average avidity of the clones selected by antigen to biosynthesise antibody as might be expected were the second phase produced by a different, or a more selected set of cells.

Another way of looking at this particular problem was to characterise the first and second phases of the adjuvant-assisted antibody response in terms of the ratio of 7S to 19S antibody, and to see if the difference in this ratio between the first and second phases resembled the difference between a primary and a secondary response following administration of antigen without adjuvant.

Experimental procedure Four birds (12 weeks of age) were each immunised by a single intramuscular injection of 5 mg HSA in Freund's complete adjuvant into the left breast muscle. The birds were bled at intervals following immunisation, and the antisera obtained from all the birds at each bleeding were pooled together. A sample from each antiserum pool was diluted 1:50, and 1 ml volumes were layered onto 4 ml sucrose density gradients which were then centrifuged overnight (as described on pages 72 and 73). After centrifugation, the polyallomer tubes were pierced and fractions were collected drop-wise

from the bottom of each tube; the amount of anti-HSA antibody in each fraction was quantitated and characterised (as described on page 73 et seq.). From this, the total amounts of 7S and of 19S anti-HSA antibody in 1 ml of undiluted antiserum were calculated; the relative proportions of 7S and 19S antibodies from both phases of the adjuvant response were compared with the proportions of 7S and 19S antibodies found in primary and secondary antibody responses elicited in a group of 3 birds immunised with 5 mg HSA in saline (iv) and challenged 40 days later with 2 mg HSA in saline (iv).

Results The results were ultimately expressed as the total amounts of 7S and 19S antibodies in 1 ml of undiluted (pooled) antiserum, and these values are given in Table 15 (a and b). The separations of 7S and 19S antibodies for each antiserum pool are shown graphically in Figure 24.

For those birds immunised with 5 mg HSA in Freund's complete adjuvant, the proportion of 19S antibody estimated in pooled antiserum 7 days after immunisation was 37.4%; this compared with a figure of 28.7% for the 19S contribution in pooled antiserum from birds 7 days after immunisation with 5 mg HSA in saline, injected intravenously. During the second phase of the adjuvant-assisted response, e.g. 28 and 34 days after immunisation, the proportion of 19S antibody was 19.5% and 16.6% respectively; this latter value corresponded to the approximate peak of the second phase of the response elicited by immunisation with antigen in Freund's complete adjuvant and was comparable to the proportion of 19S antibody (19.8%) found at the 7-day peak of a genuine anamnestic secondary response, provoked

TABLE 15a. The effect of Freund's complete adjuvant upon the relative amounts of 19S and 7S anti-HSA antibodies produced by chickens during the course of biosynthesis. Results are expressed as the total amounts (μg) of HSA bound by both 19S and 7S antibodies in 1 ml of undiluted pooled antiserum, and thence as the percentage total antibody represented by each class.

CLASS OF ANTIBODY	DAYS AFTER IMMUNISATION WITH 5 mg HSA in FCA im.		
	7	28	34
	TOTAL AMOUNT (μg) HSA BOUND BY EACH ANTIBODY CLASS IN 1 ml SERUM		
7S	23.6 (62.6%)	60.9 (80.5%)	61.7 (83.4%)
19S	14.1 (37.4%)	9.8 (19.5%)	12.2 (16.6%)

TABLE 15b. A comparison of the relative amounts of 19S and 7S anti-HSA antibodies produced by chickens at the respective 7-day peaks of a primary and a secondary antibody response. Results are expressed as above, in Table 15a.

CLASS OF ANTIBODY	7 DAYS AFTER PRIMARY IMMUNISATION WITH 5 mg HSA iv.	7 DAYS AFTER SECONDARY IMMUNISATION WITH 2 mg HSA iv.
	TOTAL AMOUNT (μg) HSA BOUND BY EACH ANTIBODY CLASS IN 1 ml SERUM	
7S	38.8 (71.3%)	47.8 (80.2%)
19S	15.5 (28.7%)	11.8 (19.8%)

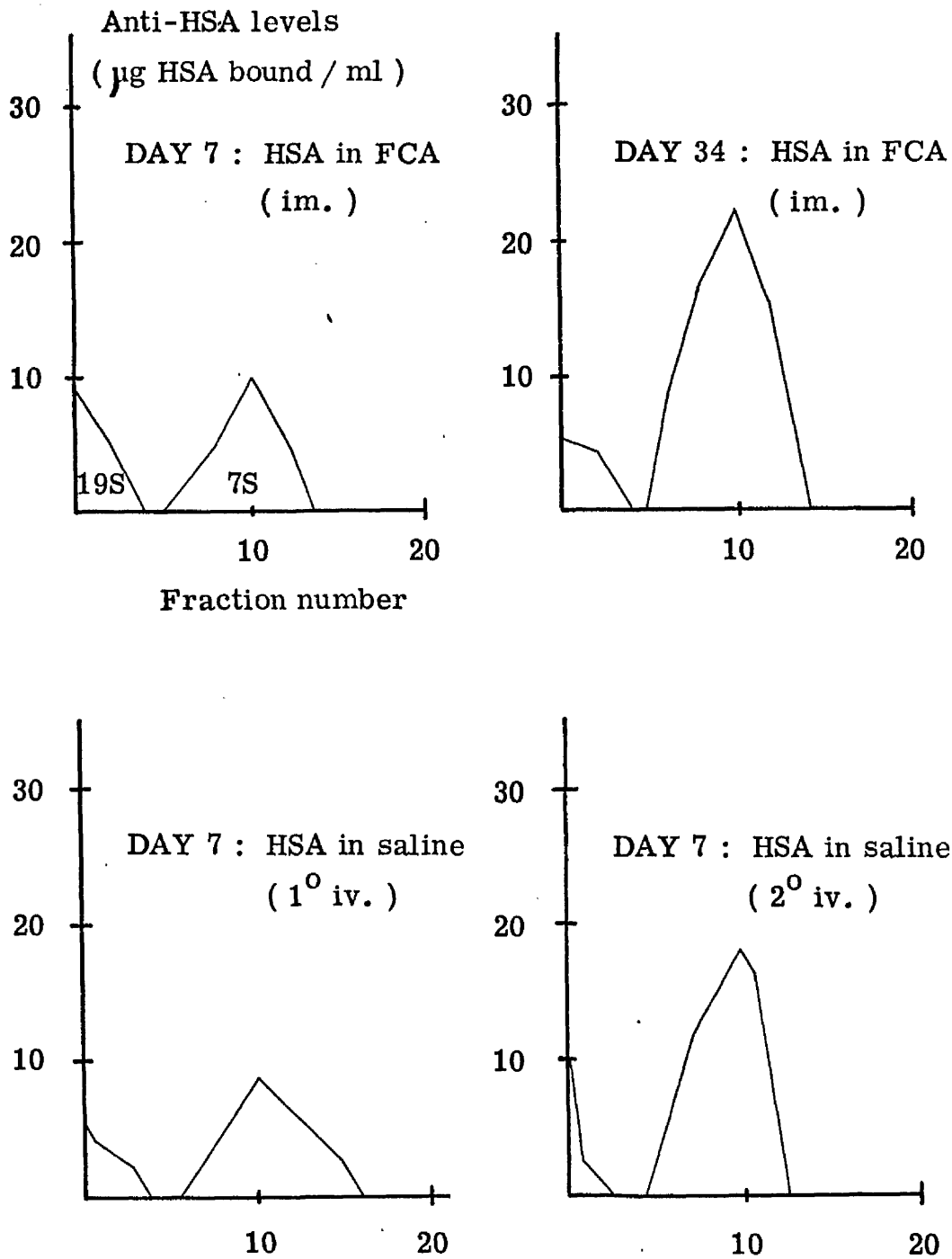


FIG. 24. Separation by sucrose density gradient ultra-centrifugation of chicken 7S and 19S anti-HSA antibodies in pooled antiserum taken 7 and 34 days after immunisation with 5 mg HSA in FCA im, also 7 days after immunisation with 5 mg HSA in saline iv and 7 days after secondary immunisation with 2 mg HSA in saline iv.

by a second injection of antigen (2 mg) given 40 days after primary immunisation.

In terms of the distribution of 7S and 19S antibodies therefore, the drop in the proportion of 19S antibody between the early and late phases of antibody production stimulated with antigen in FCA was similar to the decline in the proportion of 19S antibody between a primary and a secondary anamnestic antibody response. This was taken as evidence that specific immunological memory was imparted during the first phase of the adjuvant-assisted response, which was reflected by an increase in the 7S:19S antibody ratio in the second phase.

Experiment 10. The Effect of Bacterial Lipopolysaccharide upon the Biosynthesis of Antibodies Following Immunisation of Chickens with HSA Adsorbed onto Sheep Erythrocytes.

The biphasic antibody response observed in chickens immunised with antigen in Freund's complete adjuvant has been studied in the preceding experiments. By comparing the avidities and the respective proportions of 7S and 19S antibodies in the first and second phases it was suggested that the second phase represented in effect a secondary anamnestic response elicited by residual antigen from the water-in-oil depot at the site of injection and which perhaps followed the alleviation of feedback inhibition by the action of the adjuvant, as postulated by White (1973). A further empirical finding reported by Nielsen (1974) and by White and Nielsen (1975) was that immunisation of chickens by simultaneous injection of sheep red blood cells and Salmonella adelaide brought about a cyclical pattern of

anti-SRBC macroglobulin antibody biosynthesis; again it was postulated that the oscillating levels of antibody in the serum reflected repeated activation and relaxation of a negative feedback mechanism resulting in a regular series of ever-diminishing waves of antibody biosynthesis.

The original idea in the present experiment was to measure antibody avidity at the crest of each wave of biosynthesis throughout a cyclical antibody response, to discover whether or not there was a progressive rise in avidity. In preference to measuring avidity for such an extremely heterogeneous entity as the sheep erythrocyte, an attempt was made to elicit cyclical antibody biosynthesis to HSA; it was hoped that this could be achieved by adsorbing HSA onto the surface of sheep erythrocytes and then immunising chickens with these modified erythrocytes (HSA-SRBC) injected together with a thymus-independent antigen, bacterial lipopolysaccharide. If the production of a cyclical response to HSA was feasible, the pattern of antibody avidities from successive antibody peaks would permit further elucidation of the mechanisms of homeostasis.

Experimental procedure Two different batches of lipopolysaccharide, prepared from Escherichia coli, were kindly provided by the Department of Microbiology, Glasgow University. Three groups of chickens (12 weeks of age) were each immunised with HSA-coated tanned sheep red blood cells, prepared as described on page 112, as follows.

Group 1 (3 birds) : 10^{10} HSA-SRBC + 250 µg LPS (1) injected iv.

Group 2 (3 birds) : 10^{10} HSA-SRBC + 250 µg LPS (2) injected iv.

Group 3 (2 birds) : 10^{10} HSA-SRBC injected iv.

The birds were bled at intervals following immunisation, and anti-HSA antibodies in the serum samples were quantitated by the Farr test.

Results The results were expressed as the antigen-binding capacities for individual serum samples and these values are given in Table 16.

Although there was a good anti-HSA response in each case to immunisation with HSA-coated sheep erythrocytes, there was no evidence of cyclical anti-HSA biosynthesis - at least up until 37 days following immunisation. However, it was apparent that all the birds showed prolonged production of anti-HSA antibodies (Table 16). This result was found in all three groups showing that the effect was not dependent upon the inclusion of lipopolysaccharide with the antigen but resulted from the presentation of HSA coated onto sheep erythrocytes. Thus there were significant amounts of anti-HSA antibody detected up to 37 days after primary immunisation; also, the rate of decline of the ABC_{30} values of sera, from 3 weeks following immunisation onwards, was very slow (a virtual plateau in some cases) and was more reminiscent of the mammalian primary antibody response than the typical avian response.

There was also an apparent distinction, in terms of the absolute amounts of antibody present in the serum, between male and female birds of the same strain and age. Thus, 6 days after immunisation the average ABC_{30} for all the female birds in the experiment was 16.1 (±5.1) μg HSA ml^{-1}, whereas for all the male birds the average ABC_{30} was 71.3 (±6.4) μg HSA ml^{-1}. This finding lends

TABLE 16. The effect of *E. coli* lipopolysaccharide upon the biosynthesis of anti-HSA antibodies following immunisation of chickens with HSA-coated sheep erythrocytes. Results are expressed as the antigen-binding capacity (ABC_{30}, μg HSA ml^{-1}) of individual serum samples.

BIRD NUMBER	SEX	IMMUNISATION	DAYS AFTER IMMUNISATION 6	10	15	20	22	24	27	29	31	34	37
			SERUM ANTI-HSA ANTIBODY LEVELS; ABC_{30} (μg HSA ml^{-1})										
1221	F	GROUP 1 :	6.6	27.4	6.0	3.1	2.3	1.7	1.3	1.1	1.3	1.2	1.4
1222	F	10^{10} HSA-	22.6	13.7	7.0	5.5	6.0	4.7	4.9	4.9	5.0	5.3	4.8
1344	M	SRBC & 250 μg LPS (1) iv.	68.6	32.8	11.7	6.2	4.7	5.5	5.4	5.2	4.2	2.9	4.1
1223	F	GROUP 2 :	26.8	13.4	4.5	3.0	2.5	2.6	2.8	2.5	2.5	2.4	2.7
1224	F	10^{10} HSA-	7.9	3.1	0.8	0.9	0.9	0.8	1.0	1.0	1.0	1.1	1.0
1345	M	SRBC & 250 μg LPS (2) iv.	71.2	58.0	18.9	13.0	12.0	14.0	12.5	10.6	10.6	8.5	6.2
1346	M	GROUP 3 :	58.7	33.1	51.5	56.2	52.9	49.5	58.6	43.1	43.1	40.0	34.8
1347	M	10^{10} HSA- SRBC iv.	84.1	25.1	17.7	17.6	13.8	13.7	14.1	12.3	12.3	9.6	9.3

M - male bird

F - female bird

weight to the argument that immune responsiveness is subject to genetic control, and is maybe different for birds of different strain, sex or age.

II. Antibody-Mediated Immunosuppression as an Experimental Model of Negative Feedback Homeostasis in Chickens.

Experiment 11. The Effect of Passive Antibody and the Time of its Administration upon the Primary Antibody Response to HSA in Chickens.

Immunosuppression mediated by passive antibody has two possible modes of action; either the antibody binds to the antigen and prevents it from interacting with lymphocytes, or else the antibody and antigen act together to tolerise or incapacitate clones of antigen-sensitive cells.

In many ways the action of passively administered antibody is considered to be analogous to the homeostasis effected by antibody-mediated feedback inhibition. Therefore, the effects of passive anti-HSA upon the primary antibody response to HSA in the chicken were investigated, to find out to what extent the striking negative feedback homeostasis seen in chickens can be explained in terms of antibody-mediated feedback.

Experimental procedure Three groups of chickens (9 weeks of age) were each immunised as follows.

Group 1 : 1 ml of passive anti-HSA 24 hours before injection of 250 µg HSA iv.

Group 2 : 1 ml of passive anti-HSA 48 hours after injection of 250 µg HSA iv.

Group 3 : no passive immunisation; 250 µg HSA injected iv.

Passive antibody was prepared from pooled antiserum collected 7 days after immunisation of six 8 to 12-week old birds each with a single intravenous injection of 10 mg HSA in saline. Antibody-containing globulin was fractionated from the pooled antiserum according to the method described on pages 71 and 72. The ABC_{30} for the anti-HSA globulin was 126 µg HSA ml^{-1}.

The experimental birds were bled at intervals following active immunisation and individual serum samples were assayed for anti-HSA antibodies by the Farr test.

Results The results were expressed as the antigen-binding capacity for each serum sample and these values are given in Table 17. The kinetics of anti-HSA antibody levels in the serum of each bird are shown in Figure 25.

All the birds in Group 1 (i.e. those given passive antibody 24 hours before active immunisation) showed depressed levels of anti-HSA in the serum relative to the values obtained for birds receiving no passive antibody (i.e. those birds in Group 3). The effectiveness of passive antibody administered 48 hours after active immunisation was equivocal, as the high levels of anti-HSA in the serum of these birds (e.g. 7 days after active immunisation) could be attributed partly to residual passive antibody and partly to antibody synthesised de novo. If it is assumed that the last portion

TABLE 17. The effect of passive anti-HSA antibody and the time of its administration upon the primary antibody response to HSA in chickens. Results are expressed as the antigen-binding capacity (ABC_{30}, µg HSA ml^{-1}) of individual serum samples.

BIRD NUMBER	IMMUNISATION	DAYS AFTER ACTIVE IMMUNISATION 4	7	8	10
		SERUM ANTI-HSA; ABC_{30} (µg HSA ml^{-1})			
01	GROUP 1 :	0.5	0.7	0.6	0.2
02	Passive anti-	1.1	0.9	0.6	0.3
03	HSA 24 hours	0.4	1.1	1.0	0.4
04	before injecting	0.8	0.8	0.5	0.2
05	250 µg HSA iv.	0.6	0.7	0.6	0.2
06	GROUP 2 :	6.8	3.6	2.3	0.7
	Passive anti-				
07	HSA 48 hours	7.5	4.6	3.4	1.4
	after injecting				
	250 µg HSA iv.				
08	GROUP 3 :	0.02	1.5	1.1	0.4
09	250 µg HSA iv.	0.1	3.5	1.9	0.8
10		0.08	4.5	2.8	0.8
11		0.1	3.0	1.6	0.5

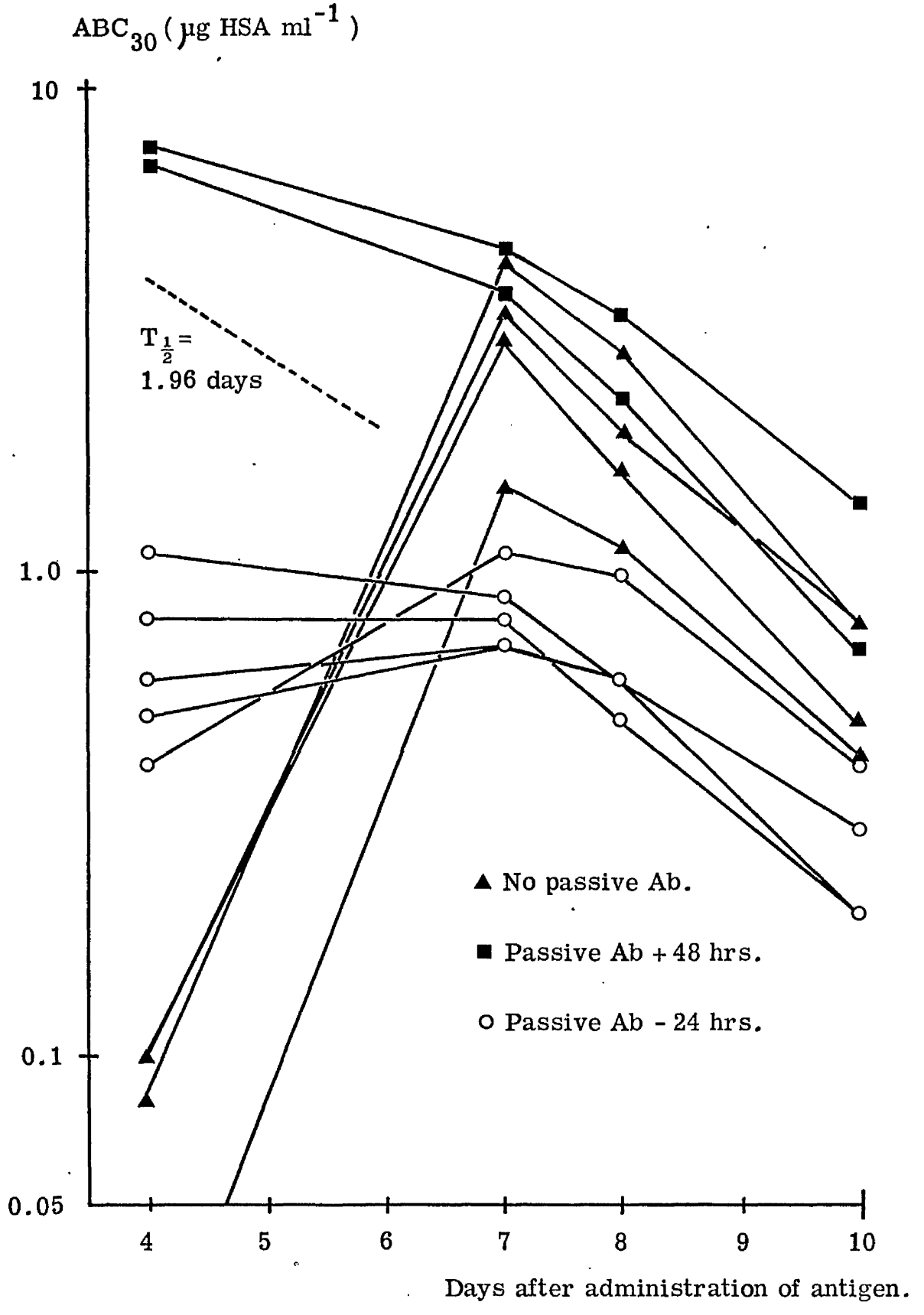


FIG. 25. The effect of passive antibody and the time of its administration upon the primary antibody response to HSA in chickens.

of the graphs in Figure 25, i.e. from day 8 to day 10, represents exponential decay of antibody without fresh synthesis, as stated by White (1973), then the half life of the antibody in the birds used in the experiment can be estimated. Visual inspection indicated a mean half life very close to 1.4 days. This would imply that in the two chickens from Group 2, which had a mean ABC_{30} of about 7.2 µg HSA ml^{-1} on day 4 (presumably all or almost all of this being passive antibody), then one would expect that by day 7 the passive antibody would have declined to an ABC_{30} of 1.6 µg HSA ml^{-1}. Thus the serum level of actively synthesised antibody can be estimated by subtracting this from the mean of the observed ABC_{30} values for day 7, which was 4.1 µg HSA ml^{-1} for the birds in Group 2. This gave a mean ABC_{30} of 4.1 - 1.6 = 2.5 µg HSA ml^{-1} which could be attributed to actively synthesised antibody. Although this is below the mean ABC_{30} (3.13 µg HSA ml^{-1}) for the control group (Group 3), it is not significantly lower (P = 0.513), making any conclusion that immunosuppression has occured in birds from Group 2 a questionable one.

Thus, passive antibody was immunosuppressive if given 1 day before antigen, but its effect was not clearly demonstrable when administered 2 days after antigen.

Experiment 12. The Effect of the Dose of Passive Antibody upon its Ability to Suppress Primary Antibody Biosynthesis in Chickens.

The results from the previous experiment established that passive antibody, administered in large amounts, was certainly capable

of immunosuppression if given before active immunisation with antigen. The quantity of antibody used in each bird to achieve this suppression of antibody biosynthesis (i.e. 1 ml of anti-HSA globulin, ABC_{30} = 126 μg ml^{-1}) was sufficient to neutralise much of the immunising dose of antigen (250 μg HSA), and it could be that the passive antibody is doing no more than lowering the effective dose of antigen. In an attempt to explore the mechanisms of antibody-mediated immunosuppression a stage further, the effect of the amount of passive antibody upon its ability to suppress antibody biosynthesis was investigated. Thus, if immunosuppression was achieved only by using large doses of passive antibody, this would indicate that the passive antibody was acting solely by neutralising the antigenic stimulus rather than having a direct effect upon the cells involved in biosynthesis.

Experimental procedure Five groups of chickens (8 weeks of age) were used in this experiment; four groups were immunised passively with chicken anti-HSA globulin and then 24 hours later the birds in all five groups were each immunised actively with 250 μg HSA. The precise details of passive immunisations, prior to the administration of antigen, are given below.

Group 1 : 1 ml passive antibody, undiluted, iv.

Group 2 : 1 ml passive antibody, diluted 1/4, iv.

Group 3 : 1 ml passive antibody, diluted 1/16, iv.

Group 4 : 1 ml passive antibody, diluted 1/64, iv.

Group 5 : no passive antibody.

The passive antibody was prepared from pooled antiserum collected 7 days after immunisation of four 15 week old birds each with a single intravenous injection of 10 mg HSA in saline. Antibody-containing globulin was fractionated as before. The ABC_{30} for the anti-HSA globulin was 202 μg HSA ml^{-1}.

The experimental birds were bled at intervals following passive and active immunisations and individual serum samples were assayed for anti-HSA antibodies by the Farr test.

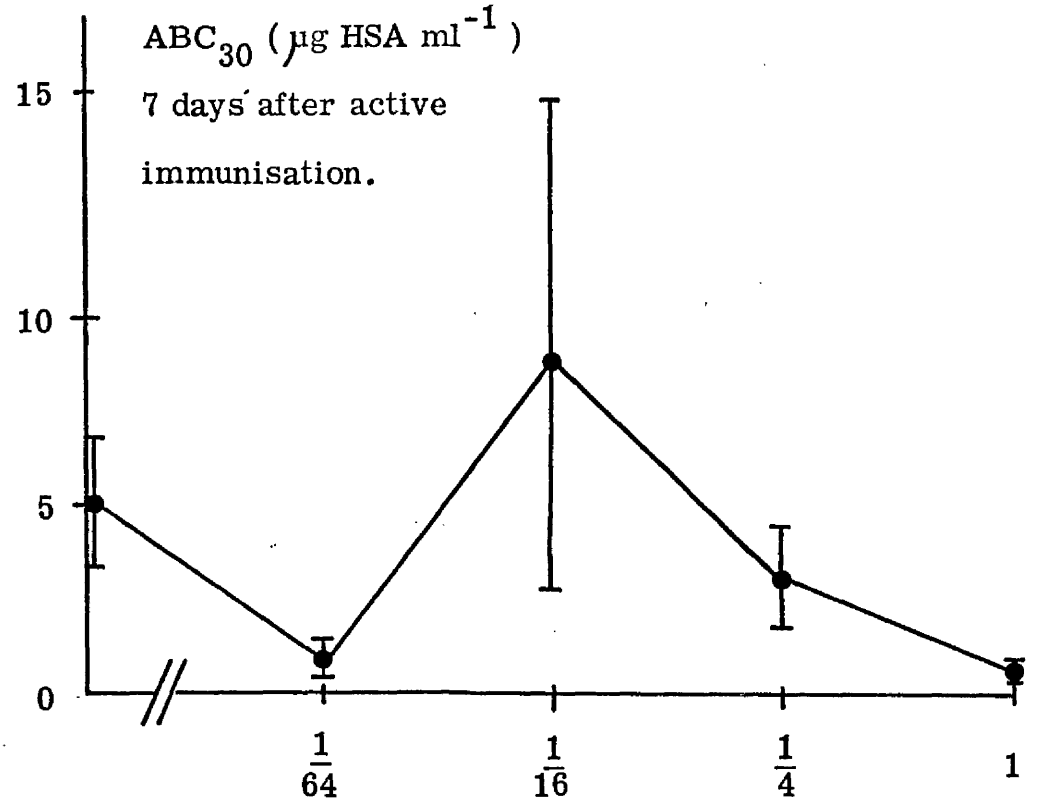
Results The results were expressed as the antigen-binding capacity for each serum sample and these values are given in Table 18; the relationship between the dose of passive antibody given and serum antibody levels 7 and 10 days after active immunisation is illustrated in Figure 26.

At 7 days after active immunisation the mean ABC_{30} values for birds in Group 1 and for birds in Group 4 were significantly lower ($P = 0.096$ and $P = 0.081$ respectively, by the two-tailed unpaired t-test) than the mean ABC_{30} for birds in the control group, Group 5. These findings suggested that, in this experiment, immunosuppression could be effected at two different dosage levels of passive antibody. In view of this, undiluted passive antibody might be expected to act by neutralising antigen in the circulation - effectively nullifying the antigenic stimulus. In contrast, such a mechanism cannot be proposed for immunosuppression achieved by passive antibody diluted 1/64, and this could reflect a direct inhibitory influence of antigen-antibody complexes (in considerable antigen excess) upon antigen-sensitive cells.

TABLE 18. The effect of the dose of passive antibody administered 24 hours before active immunisation upon its ability to suppress primary antibody biosynthesis to HSA in chickens. Serum antibody levels are expressed as the antigen-binding capacity (ABC_{30}, µg HSA ml^{-1}) for each serum sample.

BIRD NUMBER	DOSE OF PASSIVE Ab PRIOR TO 250 µg HSA iv.	DAYS BEFORE/AFTER ACTIVE IMMUNISATION -1	+3	+7	+10
		SERUM ANTI-HSA; ABC_{30} (µg HSA ml^{-1})			
25	GROUP 1 : 1 ml	19.8	0.2	1.2	1.7
26	undiluted Ab iv.	13.4	0.1	0.3	0.3
27		14.1	0.2	0.4	0.4
	Mean (±SE)	15.8(±2.0)	0.2(±0.03)	0.6(±0.3)	0.8(±0.5)
28	GROUP 2 : 1 ml	4.4	1.0	0.5	0.2
29	Ab diluted	2.5	0.03	5.7	1.4
30	1:4 iv.	3.1	0.01	5.4	3.6
34		1.9	0.01	0.9	0.4
	Mean (±SE)	3.0(±0.6)	0.3(±0.3)	3.1(±1.4)	1.4(±0.8)
31	GROUP 3 : 1 ml	1.2	0.01	3.9	1.7
32	Ab diluted	1.2	0.02	20.7	9.4
33	1:16 iv.	1.5	0.01	1.7	0.7
	Mean (±SE)	1.3(±0.1)	0.1(±0.01)	8.8(±6.0)	3.9(±2.8)
36	GROUP 4 : 1 ml	0.3	<0.01	0.4	0.3
37	Ab diluted	0.2	<0.01	1.9	1.2
38	1:64 iv.	0.3	<0.01	1.2	0.6
39		0.4		1.3	0.6
	Mean (±SE)	0.3(±0.05)	nd	1.2(±0.3)	0.7(±0.2)
40	GROUP 5 :	nd	<0.01	8.4	3.9
41	No Ab.	nd	<0.01	1.4	0.9
42		nd	<0.01	2.5	1.9
43		nd	<0.01	8.0	3.3
	Mean (SE)	nd	nd	5.1(±1.8)	2.5(±0.7)

nd - not determined



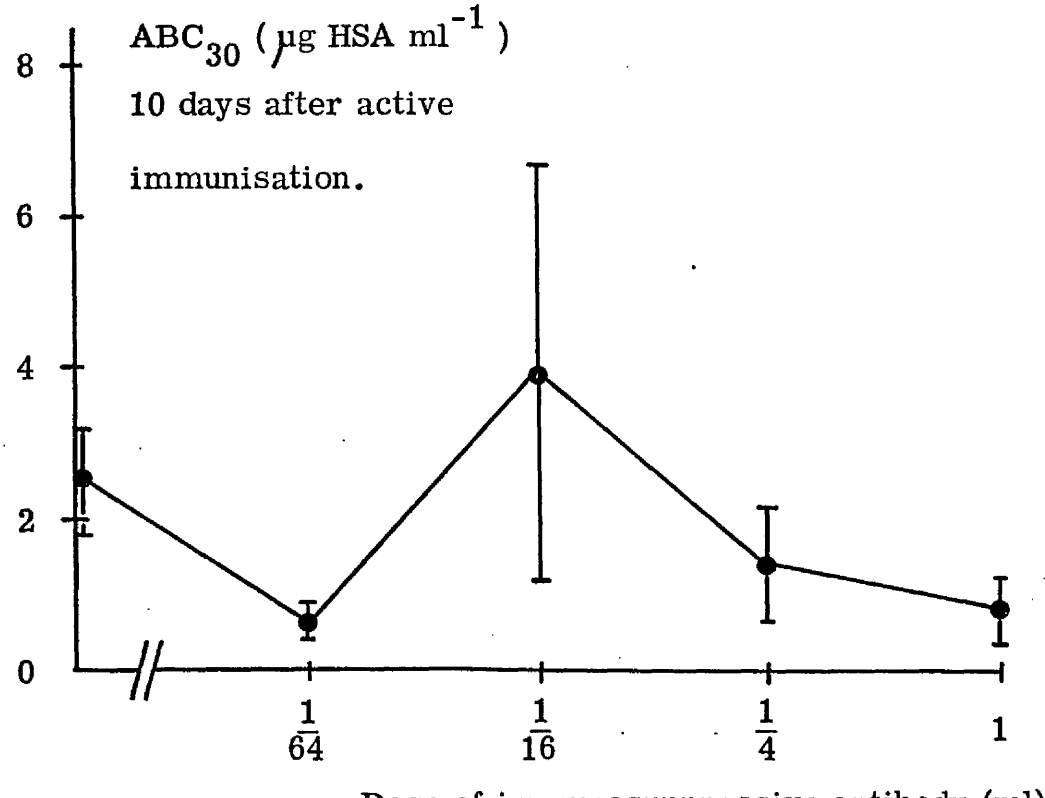


FIG. 26. The effect of the dose of passive antibody (given 24 hours before antigen) upon its ability to suppress primary antibody biosynthesis to HSA in chickens.

Experiment 13. Comparison of the Immunosuppressive Potency of Chicken Antibody Obtained either from Serum 7 Days after Primary Immunisation or Extracted from Freund's Adjuvant Granulomata 48 Days after Immunisation.

It has been found in mammals that the degree of immunosuppression achieved by equivalent amounts of passive antibody was directly related to the affinity of the antibody (e.g. Walker and Siskind, 1968). An attempt was therefore made to correlate the immunosuppressive potency with the avidity of passive antibody in chickens.

The respective sources of low and high avidity chicken anti-HSA antibodies were the serum obtained at the 7-day peak of the primary antibody response, and the extract from granulomata obtained at the 48-day peak of the second phase of the adjuvant-assisted response in chickens. The granuloma extract was used as a source of high avidity anti-HSA since French, Stark and White (1970) demonstrated that antibody from the second phase of the antibody response in chickens following immunisation with antigen in Freund's complete adjuvant was more avid than that from the initial phase.

The present experiment was therefore conducted to test the hypothesis that high avidity antibody is more immunosuppressive than an equivalent amount of low avidity antibody. Thus the immunosuppressive capabilities of antibody from the peak of the primary antibody response in chickens were compared with those of antibody contained in granuloma extract.

Experimental procedure Two groups of chickens (6 weeks of age) were each given an intravenous injection of one of the passive antibody preparations followed 24 hours later by immunisation with HSA. A third experimental group of birds received no passive antibody but were immunised with HSA. The schedule for immunisation was as follows.

Group 1 : 1 ml passive anti-HSA (peak primary response) iv. 24 hours before injection of 250 µg HSA iv.

Group 2 : 1 ml passive anti-HSA (granuloma extract) iv. 24 hours before injection of 250 µg HSA iv.

Group 3 : no passive antibody; active immunisation with 250 µg HSA iv.

Passive anti-HSA globulin was obtained from two sources : (1) from pooled antiserum collected 7 days after immunisation of three 12-week old birds each with a single intravenous injection of 2 mg HSA in saline. The ABC_{30} was 10.3 µg HSA ml^{-1}. (2) from granuloma extract obtained 48 days after immunisation of four 8-week old chickens with 5 mg HSA in FCA. The ABC_{30} was 11.4 µg HSA ml^{-1}.

The experimental birds were bled at intervals following passive and active immunisations and individual serum samples were assayed for anti-HSA by the Farr test.

Results The results were expressed as the antigen-binding capacity for each serum sample and these values are given in Table 19. There was no apparent difference in the amount or kinetics of antibody biosynthesis between any of the 3 experimental groups of birds,

TABLE 19. Comparison of the immunosuppressive potency of chicken antibody obtained either from serum 7 days after primary immunisation or extracted from Freund's adjuvant granulomata 48 days after immunisation. Serum antibody levels are expressed as the antigen-binding capacity (ABC_{30}, µg HSA ml^{-1}).

BIRD NUMBER	IMMUN-ISATION	DAYS AFTER ACTIVE IMMUNISATION 3	7	12	21
		SERUM ANTI-HSA; ABC_{30} (µg HSA ml^{-1})			
399	GROUP 1 :	0.6	7.3	2.5	1.4
401	1 ml Ab (1°,	0.5	11.1	3.9	1.5
425	day 7) 24 hours	1.0	9.5	2.6	1.8
431	before 250 µg HSA iv.	0.9	11.4	3.7	2.2
	MEAN (±SE)	0.8(±0.1)	9.8(±0.9)	3.2(±0.3)	1.7(±0.2)
407	GROUP 2 :	1.2	9.2	3.7	3.7
402	1 ml Ab (granuloma	0.8	8.5	3.0	1.7
411	extract) 24	1.0	12.2	6.4	2.4
416	hours before 250 µg HSA iv.	0.9	8.8	3.2	2.9
	MEAN (±SE)	1.0(±0.1)	9.7(±0.8)	4.1(±0.8)	2.7(±0.4)
418	GROUP 3 :	1.1	3.9	2.0	1.3
419	No Ab; 250 µg	1.5	7.5	3.6	1.6
421	HSA iv.	0.9	8.1	4.7	2.9
429		0.6	10.4	3.4	2.0
	MEAN (±SE)	1.0(±0.2)	7.5(±1.4)	3.4(±0.5)	2.0(±0.3)

including the control group. This obviously reflected a failure to achieve immunosuppression by antibody prepared either from primary immune serum or from granuloma extract. The quantity of antibody in these preparations was too small to have much neutralising effect on antigen in vivo, nor was there any evidence of immunosuppression having been achieved by mechanisms other than straightforward neutralisation, as was found, for example, in Experiment 12.

Experiment 14. The Effect of Passive Anti-Carrier and Anti-Hapten Antibodies upon the Primary Antibody Response of Chickens to a Hapten-Protein Conjugate.

The experimental investigation of antibody-mediated immunosuppression in chickens has so far been concerned with the level at which passive antibody operates, whether it is by neutralising antigenic determinants or else by inhibiting the cells responsible for the biosynthesis of antibody, or both.

Another clue to this puzzle comes from the determinant specificity of antibody-mediated immunosuppression. As mentioned on pages 42 and 43, it has been found in certain instances that immunosuppression was absolutely determinant specific (i.e. that antibody to one determinant suppressed the response to that determinant only) whilst in other cases the specificity overlapped to include unrelated determinants on the same antigen. The present experiment was to examine the effects of either anti-carrier or anti-hapten antibodies, given passively, upon the antibody response of chickens to the hapten and to the carrier components of a hapten-protein conjugate.

Experimental procedure Three groups of chickens (15 weeks of age) were each immunised as follows.

Group 1 : 1.3 ml of anti-HSA globulin iv. 12 hours before injection of 500 μg DNP_{12}-HSA iv.

Group 2 : 1.3 ml of anti-DNP globulin iv. 12 hours before injection of 500 μg DNP_{12}-HSA iv.

Group 3 : no passive antibody before injection of 500 μg DNP_{12}-HSA iv.

Passive antibody was obtained from 2 separate pools of antiserum, each collected 7 days after immunisation of 2 groups of three chickens (15 weeks old) immunised with a single intravenous injection of 5 mg HSA or 2 mg DNP-HCN in saline respectively. The ABC_{30} for anti-HSA globulin was 150 μg HSA ml^{-1}; HBC_{30} for anti-DNP globulin was 6240 ng DNP-lysine ml^{-1}. The experimental birds were bled at intervals following passive and active immunisations and individual serum samples were quantitated for anti-HSA and anti-DNP antibodies by separate Farr tests.

Results The results were expressed as the DNP- and HSA-binding capacities of each serum sample and these values are given in Table 20. There was no evidence of immunosuppression mediated by passive antibody, in that birds belonging to Groups 1 and 2, immunised passively with anti-HSA and anti-DNP respectively, produced antibodies to DNP-HSA in comparable amounts to those produced by the control birds in Group 3, which received no passive antibody. The moderate differences which did occur were no larger than might be expected to arise by chance, as judged by Student's t-test.

TABLE 20. The effect of passive anti-carrier and anti-hapten antibodies upon the primary antibody response of chickens to a hapten-protein conjugate (DNP-HSA). Results are expressed as (a) anti-HSA levels in terms of the antigen-binding capacity (ABC_{30}, µg HSA ml^{-1}) and (b) anti-DNP antibody levels in terms of the hapten-binding capacity (HBC_{30}, ng DNP-lysine ml^{-1}).

BIRD NUMBER	IMMUNISATION	TIME AFTER IMMUNISATION WITH DNP-HSA 30'	5 days	7 days	10 days
		ANTI-HSA LEVELS; ABC_{30} (µg HSA ml^{-1})			
1201	GROUP 1: anti-	0.9	0.1	1.8	1.1
1202	HSA 12 hours	0.8	2.5	36.1	8.9
1203	before 500 µg DNP-HSA iv.	1.0	1.8	8.5	3.6
	Mean (±SE)	0.9(±0.06)	1.5(±0.7)	15.5(±10.5)	4.5(±2.3)
1204	GROUP 2: anti-	0.06	2.2	7.0	2.5
1205	DNP 12 hours	0.08	25.4	54.4	24.5
1206	before 500 µg DNP-HSA iv.	0.06	4.8	15.3	7.3
	Mean (±SE)	0.07(±0.01)	10.8(±7.3)	25.6(±14.6)	11.4(±6.7)
1207	GROUP 3:	nd	8.1	26.7	9.9
1208	500 µg DNP-HSA	nd	3.4	4.0	1.2
1209	iv.	nd	42.5	52.4	14.8
	Mean (±SE)	nd	18.0(±12.3)	27.7(±14.0)	8.6(±3.9)

BIRD NUMBER	IMMUNISATION	TIME AFTER IMMUNISATION WITH DNP-HSA 30'	5 days	7 days	10 days
		ANTI-DNP LEVELS; HBC_{30}(ng DNP-lys ml^{-1})			
1201	GROUP 1: anti-	12.6	24.6	44.1	32.1
1202	HSA 12 hours	23.4	101.6	161.7	78.8
1203	before 500 µg DNP-HSA iv.	24.8	40.5	96.4	51.1
	Mean (±SE)	20.3(±3.9)	55.6(±23.5)	100.7(±34.0)	54.0(±13.6)
1204	GROUP 2: anti-	99.7	84.3	117.5	64.8
1205	DNP 12 hours	194.0	196.1	275.5	105.1
1206	before 500 µg DNP-HSA iv.	137.0	169.9	203.6	90.9
	Mean (±SE)	143.6(±27.4)	150.1(±33.8)	198.9(±45.6)	86.9(±11.8)
1207	GROUP 3:	nd	123.6	216.5	81.8
1208	500 µg DNP-	nd	52.7	49.6	25.4
1209	HSA iv.	nd	96.3	87.2	43.1
	Mean (±SE)	nd	90.9(±20.7)	117.8(±50.5)	50.1(±16.6)

nd - not determined

In this experiment there was enough anti-HSA administered to birds in Group 1 to bind about 40% of the HSA injected subsequently; and there was enough anti-DNP administered to bind about 20% of the DNP, i.e. about 2 DNP groups per molecule of conjugate. The results show that this was insufficient to produce a demonstrable immunosuppressive effect. This tends somewhat to support the hypothesis that antibody-mediated immunosuppression in chickens depends on neutralisation of the antigenic stimulus, rather than suppression of lymphocytes by complexes in antigen excess.

Experiment 15. The Effect of the Repeated Injection of Antigen upon the Magnitude and Duration of Serum Antibody Levels in the Chicken. An Attempt to Delay Feedback Inhibition.

The prompt switching off of antibody biosynthesis in the chicken at about 7 days after immunisation has been attributed to efficient feedback regulation by circulating antibody (White, 1973) - and Uhr and Möller (1968) have emphasised the analogy between antibody-mediated immunosuppression and homeostasis by feedback inhibition.

In the present experiment a further attempt was made to distinguish between the possible mechanisms of antibody-mediated immunosuppression. If the mechanism is solely the neutralisation of the antigenic stimulus, it would be expected that the negative feedback could be prevented or at least delayed by administering more and more antigen during the first four days of antibody biosynthesis. On the other hand, if the immunosuppressive agent is an antigen-antibody complex formed in antigen excess, then its action should not be so

easily inhibited by repeated administration of antigen.

Experimental procedure Three groups of chickens (20 weeks old) were each immunised as follows.

Group 1 : Primary immunisation with 5 mg DNP_{12}-HSA iv. This was followed by further injections of 1 mg HSA iv. 3, 4, 5, 6 and 7 days later.

Group 2 : Primary immunisation with 5 mg DNP_{12}-HSA iv. This was followed by further injections of 40 µg of the divalent DNP hapten 1,6-bis-(2,4-dinitrophenylamino)hexane iv. 3, 4, 5, 6 and 7 days later.

Group 3 : Primary immunisation with 5 mg DNP_{12}-HSA iv. only.

The birds were all bled at intervals following primary immunisation with DNP-HSA; on those days when both bleeding and administration of antigen supplements had to be done, the blood samples were taken first. Individual serum samples were assayed for anti-HSA and anti-DNP antibodies by Farr tests.

Results The results were expressed as the separate DNP-binding and HSA-binding capacities of each serum sample, and these values are given in Table 21.

Supplementary injections of HSA, given for 5 consecutive days beginning 3 days after the initial immunisation with DNP-HSA, caused an increase in the levels of anti-HSA detected and also caused a few days delay in the attainment of peak levels of anti-HSA in the serum of 3 out of 4 birds in the group, as shown in Figure 27. This procedure however had no effect upon the amount or time of synthesis of anti-DNP antibody in comparison to the control group of birds. In contrast, supplementary injections of a divalent hapten (DNP_2-

TABLE 21. The effect of priming chickens with DNP-HSA (5 mg iv.) and then repeatedly boosting on days 3, 4, 5, 6 & 7 with either HSA or with divalent DNP hapten. Results are expressed as (a) anti-HSA antibody levels in terms of the antigen-binding capacity (ABC_{30}, μg HSA ml^{-1}) and (b) anti-DNP antibody levels in terms of the hapten-binding capacity (HBC_{30}, ng DNP-lysine ml^{-1}).

BIRD NUMBER	SEX	IMMUNISATION	DAYS AFTER FIRST IMMUNISATION 5	7	10	14
			ANTI-HSA LEVELS; ABC_{30} (μg HSA ml^{-1})			
1210	F	GROUP 1: 5 mg	39.8	290.4	93.4	24.5
1211	F	DNP-HSA iv,	0.2	67.2	164.7	50.5
1212	F	then 1 mg HSA	8.6	247.2	355.5	75.5
1218	M	iv. 3,4,5,6 & 7 days later	0.1	1.0	41.6	7.2
		Mean (±SE)	12.2(±9.4)	151.5(±69.7)	163.8(±63.7)	39.4(±14.9)
1213	F	GROUP 2: 5 mg	50.2	126.1	22.7	6.3
1214	F	DNP-HSA iv,	31.8	59.1	19.7	7.1
1215	F	then 40 μg	110.9	77.9	24.9	9.2
1219	M	divalent DNP iv. 3,4,5,6 & 7 days later	0.7	0.7	1.5	0.3
		Mean (±SE)	48.4(±23.2)	65.5(±25.9)	17.2(±5.3)	5.7(±1.9)
1216	F	GROUP 3:	5.0	21.6	6.5	2.6
1217	F	5 mg DNP-HSA	11.3	30.5	5.7	*
1220	M	iv.	0.3	1.8	0.9	0.5
		Mean (±SE)	5.5(±3.2)	18.0(±8.5)	4.4(±1.7)	1.6(±1.1)
			ANTI-DNP LEVELS; HBC_{30} (ng DNP-lys ml^{-1}			
1210	F	GROUP 1: 5 mg	69.6	66.5	51.9	23.0
1211	F	DNP-HSA iv.	184.1	375.3	134.7	57.3
1212	F	then 1 mg HSA	224.6	269.7	151.6	64.0
1218	M	iv. 3,4,5,6 & 7 days later	97.9	145.8	94.8	110.4
		Mean (±SE)	144.1(±36.2)	214.3(±68.0)	108.3(±22.2)	63.7(±18.0)
1213	F	GROUP 2: 5 mg	173.3	273.1	103.8	48.3
1214	F	DNP-HSA iv,	169.8	348.4	123.8	59.0
1215	F	then 40 μg	275.2	235.3	95.1	50.6
1219	M	divalent DNP iv. 3,4,5,6 & 7 days later	37.5	36.5	19.3	21.3
		Mean (±SE)	151.5(±40.0)	223.3(±62.5)	85.5(±22.8)	44.8(±8.2)
1216	F	GROUP 3:	266.9	510.8	204.9	97.2
1217	F	5 mg DNP-HSA	107.0	182.9	42.2	*
1220	M	iv.	87.3	243.9	209.5	192.1
		Mean (±SE)	153.7(±57.0)	159.5(±56.8)	152.2(±55.1)	144.7(±47.6)

M - male bird; F - female bird; * - bird died

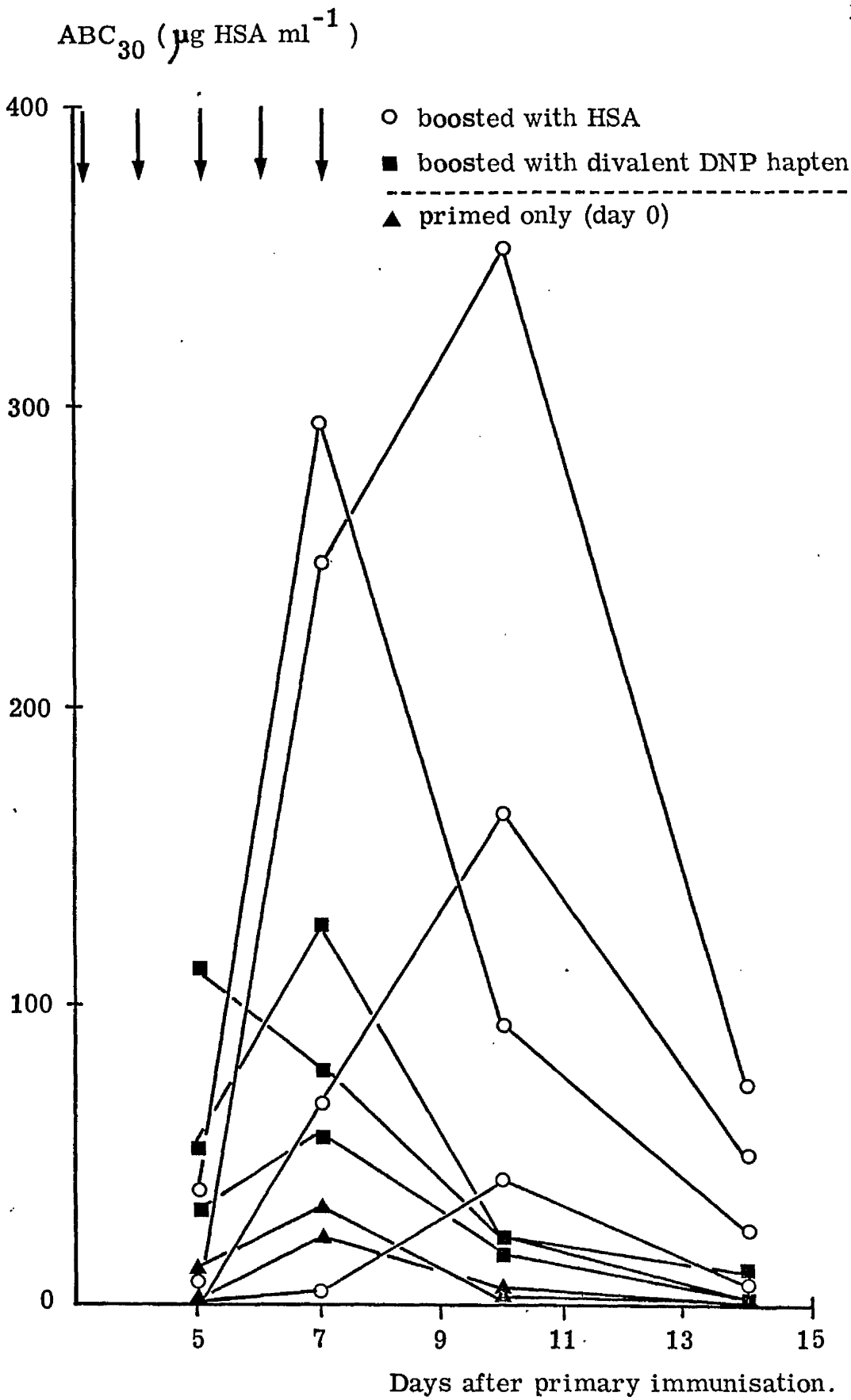


FIG. 27. The effect of repeated injection of antigen (either HSA or divalent DNP hapten) upon the magnitude and duration of anti-HSA antibody levels in chickens primed with DNP-HSA.

diaminohexane) had no effect either on the amount of anti-DNP produced, or upon the time at which peak levels of anti-DNP were detected in the serum relative to the control group of birds (i.e. day 7).

Although there was evidence that supplementary injections of HSA, as well as eliciting increased amounts of antibody, caused an apparent delay in the arrival at peak levels of anti-HSA in the serum (until day 10 for 3 out of 4 birds in the group, $\chi^2 = 3.96$; $P = 0.026$), this result must be interpreted with caution. In this experiment the antigen-binding capacities do not reflect merely the synthesis of antibody, but are also affected by its neutralisation and removal by the repeated injections of antigen. It was however possible to make some approximate calculations as to what extent the repeated injection of antigen affected the ABC_{30} value. For example, if a 20-week old bird has a body weight of about 2 kg and therefore a total blood volume of 200 ml, then by day 7 birds in Group 1 would each have received a total of 4 mg HSA in addition to the original injection of 5 mg DNP-HSA; that is the ABC_{30} values recorded at day 7 would have been effectively lowered by as much as 20 $\mu g\ ml^{-1}$ due to repeated injection of antigen. Even making this allowance, 3 out of 4 birds in Group 1 still had a higher serum ABC_{30} value at 10 days than at 7 days, suggesting that biosynthesis had not been completely switched off.

The production of antibody by individual birds was again found to depend on the sex of the bird; in this case the sera of female birds contained much greater amounts of anti-HSA and slightly increased amounts of anti-DNP antibodies compared with serum samples from male birds. This observation contrasts with that reported on page 167, where male birds produced appreciably more antibody than female birds.

Experiment 16. Screening of Chicken Anti-HSA Antisera for Evidence of Anti-Idiotype Autoantibodies.

The antibody contributing to the negative feedback of a response to a given antigen has usually been thought to be specific for that antigen, but there has been recent evidence (Fitch, 1974) that it may in fact be an autoantibody specific for an immunoglobulin idiotype which in turn is specific for the antigen. If this were true in chickens, it would be expected that serum obtained early in an immune response, containing the first idiotypes formed, would react with serum obtained from the same individual later in the response when anti-idiotype antibody may have been formed. The aim of this experiment was to search for evidence of such reactions.

Experimental procedure Six birds (15 weeks of age) were each immunised with 10 mg HSA in saline, a single intravenous injection. The birds were bled 5, 6, 7, 9, 10, 11 and 14 days after immunisation.

The sera obtained from individual birds at various times after immunisation were then tested in a double diffusion gel for evidence of autoantibody activity. Each antiserum from an individual was tested in turn against the other six antiserum samples from the same individual, by placing about 2 µl of the antiserum in a central well with the remaining antisera in six equally-spaced wells surrounding (and 10 mm from) this central well. All tests were done at two different salt concentrations; i.e. in agar gel containing 0.8% (0.137 M) NaCl as well as in agar gel containing 8% (1.37 M) NaCl. This was done to exclude the possibility of chicken antibodies failing to precipitate

at physiological salt concentrations, as has been reported by Hersh and Benedict (1966).

The double diffusion gels were left at room temperature for 48 hours and then examined for evidence of autoantibody activity, in the form of precipitin lines, between antisera taken early during the antibody response and antisera collected at later stages in the response.

Results Precipitin lines were seen in the gel only for tests involving antiserum samples from one individual bird. In this case, antiserum taken 5 days after immunisation appeared to react with antiserum from the same bird taken 7, 9 and 10 days after immunisation. However, this reaction reflected the presence of residual amounts of HSA in the serum of the bird 5 days after immunisation, which reacted in the gel with the anti-HSA antibodies present 7, 9 and 10 days after immunisation, rather than demonstrating the presence of anti-idiotype autoantibodies.

The bird in question had the highest ABC_{30} value at day 7 of the group of six, and in fact serum (representing days 7, 9 and 10) from this bird reacted similarly (i.e. formed weak precipitin lines) when tested in a double diffusion gel with day 5 antisera from all the other birds in the group (at both high and low salt concentrations).

There was therefore no evidence of anti-idiotype autoantibodies being produced and acting to regulate continued biosynthesis of specific antibody by the individual.

DISCUSSION

Among the several factors which control or can influence an immune response, the nature of the antigenic stimulus (for example, the type and dose of antigen) is one of the most important considerations, and affects antibody biosynthesis from its inception. In addition there are other factors which may modify or regulate an immune response, including for instance the use of adjuvants and the operation of homeostatic mechanisms.

The physical and chemical characteristics of an antibody response are useful indicators of how that response is being controlled. These indications include not only the kinetics of biosynthesis but also certain attributes of the antibody molecules themselves - their specificity, affinity, avidity, as well as their class and the heterogeneity of affinities within a population; all of these parameters show considerable variability under experimental conditions, thus affording a means of studying the cellular and molecular events which constitute an immune response and the mechanisms by which it is controlled.

CONTROL RELATED TO IMMUNOGENICITY

The selection of lymphoid precursor cells by antigen was studied by following the kinetics of antibody biosynthesis and then interpreting experimental results in terms of the induction and subsequent regulation of the biosynthesis of specific antibody.

The kinetics of antibody biosynthesis in chickens immunised with a single intravenous injection of HSA in saline showed

that there was a rapid rise in serum antibody levels between 4 and 7 days following immunisation, and after the peak level had been reached (about the seventh day) there was a steady exponential decline in the amount of antibody detected in the serum; these findings are apparent from Table 4 (page 118) and are shown in Figure 15 (page 119) and were in agreement with the results of White, French and Stark (1970). Since chicken IgG has a relatively short half-life, for example a value of 1.93 days has been cited by White (1973), this means that the rise and fall of serum antibody levels quickly reflect the preceding events at the cellular level with reasonable accuracy. The early pattern of antibody production can therefore be reconciled with an initial recruitment of lymphoid precursor cells as a result of antigen molecules interacting with cell-bound receptors of complementary specificity, followed by differentiation and proliferation of these triggered cells. If the interaction between antigen and cell-associated receptors is at all analogous to the interaction between antigen and antibody *in vitro*, then clearly this would not be a rate-limiting step in the production of antibody. The interval of approximately 4 days between the administration of antigen and the detection of free antibody in the circulation must be determined by the rate at which activated cells differentiate, proliferate and start secreting antibody molecules in detectable amounts; it is possible that attributing a time lag of 4 days to the processes of differentiation and proliferation is an exaggeration - due to the fact that the first antibody molecules to be produced by plasma cells will associate with antigen in the circulation and will therefore evade detection by an antigen-binding assay.

More direct evidence for the sequence and timing of events in the induction phase came from studying the population kinetics of specific plasma cells in the splenic red pulp of chickens immunised with 10 mg HSA, as reflected by the number of immunofluorescent antibody-containing cells per unit area at various times after immunisation. Plasma cells secreting anti-HSA antibodies were first detected in the red pulp of the spleen 3 days after immunisation, their number rising sharply to a maximum at 4 days and subsequently diminishing so as to be virtually absent 8 days after immunisation (Table 5, page 121; Figure 16, page 122); the kinetics of the plasma cell population followed the same pattern as the kinetics of antibody levels in the serum (Figure 15, page 119) but there were temporal discrepancies, for example between the time at which specific anti-HSA plasma cells were first detected (3 days) and the apparent onset of antibody biosynthesis (4 days), or between the time at which the density of specific plasma cells in the spleen was at its greatest (4 to 5 days) and the attainment of peak antigen-binding capacities in the serum (about 7 days). Perhaps the first mentioned of these discrepancies could be explained by the limitations of an antigen-binding assay for representing actual biosynthesis of antibody at a time when the vast majority of the specific antibody molecules secreted by plasma cells are being neutralised by combination with antigen in the body, as was suggested previously. In support of this, White, French and Stark (1970) have shown that in chickens immunised with HSA, circulating HSA:anti-HSA complexes were detectable at least 1 day before any antigen-binding by the serum was evident, and that as much as 10% of a 10 mg dose of HSA injected

intravenously was still in the circulation 4 days after immunisation. Although White, French and Stark (1970) have also reported that the maximum number of antibody-containing cells in the red pulp was not reached until 6 days after immunisation, it must be pointed out that in their experiments peak antibody levels in the chickens were not attained until 9 days after immunisation.

The response of chickens to the DNP hapten was studied for two reasons: firstly, to find out what features the kinetics of the anti-DNP antibody response had in common with the kinetics of the anti-HSA response, and secondly, depending upon the capability of chickens to biosynthesise anti-DNP antibodies, to measure the affinity of anti-DNP antibodies produced under various experimental conditions.

Although the production of anti-hapten antibodies by chickens has been reported in the past (Gold and Benedict, 1962; Orlans, Saunders and Rose, 1968; Gallagher and Voss, 1969; Voss and Eisen, 1972; Sarvas et al., 1974; Yamaga and Benedict, 1975a), there has been no quantitative examination of the kinetics of anti-hapten antibody biosynthesis in chickens. Orlans, Saunders and Rose (1968) have in fact emphasised how surprisingly difficult it was to elicit an antibody response in chickens to a haptenated protein (DNP-BGG), to the extent that they resorted to intra-splenic administration of the antigen in trying to elicit biosynthesis of anti-DNP antibodies. Furthermore, Yamaga and Benedict (1975a) have reported that chickens given a single intravenous injection of 2 mg DNP-BGG did not produce enough antibody for equilibrium dialysis measurements, even though both 7S and macroglobulin anti-DNP antibodies were detected by radio-immunoelectrophoresis.

The importance of the antigen, especially the nature of the carrier to which haptens are attached, in controlling the course of biosynthesis was explained in the Introduction (page 39). The early theories, such as postulated by Singer (1964), that the carrier did no more than provide a suitably immunogenic local environment for each haptenic determinant have largely been superseded, and it is now considered more likely that the carrier is recognised by specific "helper" cells (probably T lymphocytes) and that this recognition somehow facilitates the activation of hapten-specific antibody-producing cells (Mitchison, 1969; Rajewsky, 1971).

From the early work of Landsteiner and Simms (1923), reinforced by more recent investigations (e.g. Siskind, Paul and Benacerraf, 1966), it was concluded that to elicit biosynthesis of antibodies specific for a haptenic determinant, the carrier to which that hapten was attached had itself to be immunogenic. This prompted the trial of three different carriers for their suitability to promote antibody responses in chickens to the DNP which was conjugated to each carrier: the carriers investigated were (1) A large protein molecule, haemocyanin (MW about 7×10^6 daltons) from Helix pomatia. (2) A particulate carrier in the form of human erythrocytes. (3) A structural polysaccharide of regularly repeating units, which in this instance was chromatographic cellulose. From the small but significant increase in anti-DNP levels detected in serum samples between 3 and 7 days after immunisation of chickens with DNP groups coupled to either haemocyanin or human erythrocytes (Table 6, page 126), it was apparent that these two entities had some proficiency as

carriers for the hapten, although the amount of anti-DNP antibody produced in either case was small. DNP coupled to chromatographic cellulose elicited no antibody formation (Table 6, page 126); although the exact cause for this was not apparent, perhaps the lack of intrinsic immunogenicity invalidated this particular molecule as a suitable carrier.

Another aspect of the chemical nature of the antigen which in other animal models has been found to influence production of anti-hapten antibodies, is the degree of substitution of haptenic determinants on the carrier. If co-operation between carrier-specific "helper" T cells and hapten-specific antibody-producing B cells can be envisaged as a means for effectively concentrating antigen sufficiently for triggering the hapten-specific B cells, as suggested by Mitchison (1969), then a high degree of hapten substitution on the carrier might be expected to increase the local concentration of hapten even more and improve the chances of successful activation. It has in fact been shown in mammals by Kontiainen (1971), Larralde and Janof (1972) and by Davie and Paul (1972a) that multi-substituted antigens were more effective than oligo-substituted antigens for elicitation of high titre anti-hapten antibodies. On this evidence there appeared to be a direct but non-linear relationship between the degree of hapten substitution (allowing for the increase in the absolute concentration of hapten) and the amount of anti-DNP which was produced by the animal. This relationship is illustrated in Figure 28 which is based on the observations of Larralde and Janof (1972) and the same authors also demonstrated that antibody affinity was inversely related to the number of hapten groups per carrier molecule.

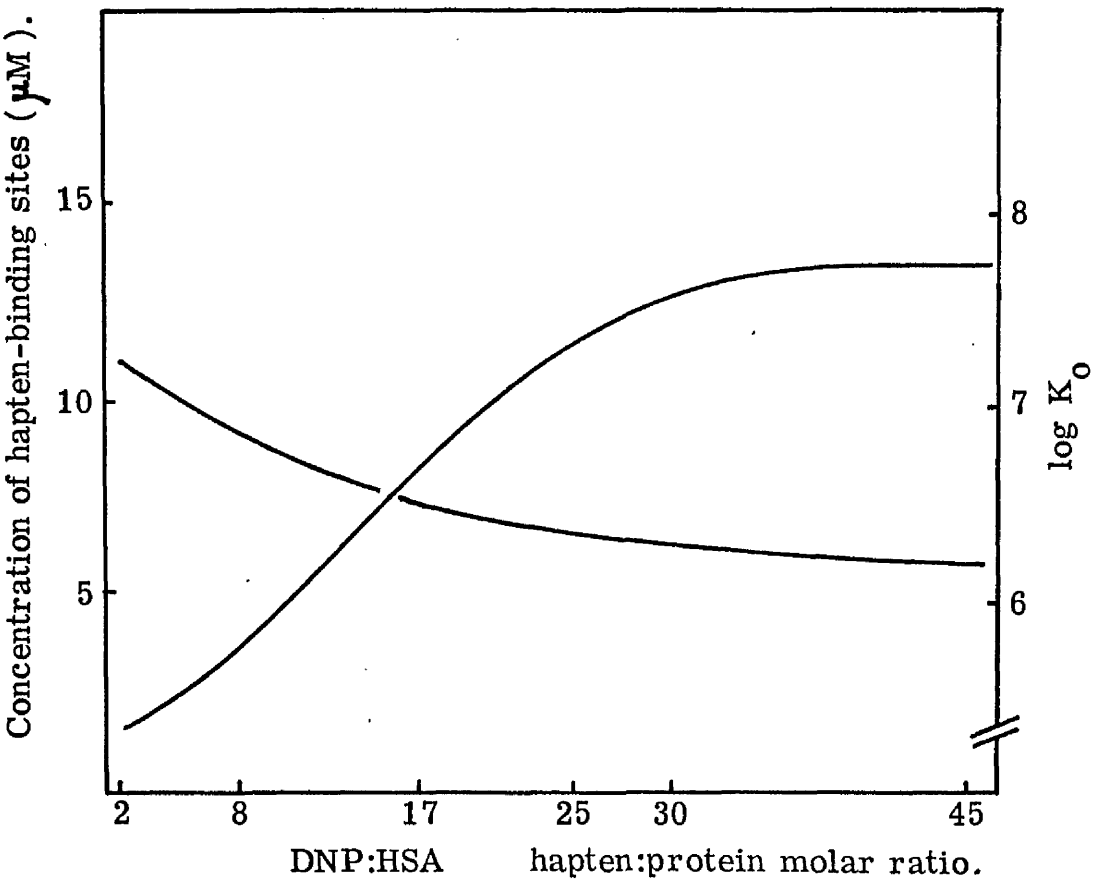


FIG. 28. The effect of the hapten-carrier molar ratio upon the quantity and the affinity of anti-hapten antibody produced, (after Larralde and Janof, 1972).

Attempts to relate, by experiment, the amount of anti-DNP produced by chickens to the degree of haptenic substitution of the immunogen, furnished inconclusive results. Thus neither densely (DNP_{43}-HSA) nor sparsely (DNP_6-HSA) haptenated carrier protein provoked biosynthesis of anti-DNP antibodies. However it was interesting to note that levels of anti-HSA (i.e. antibodies directed against the carrier) were inversely related to the epitope density (Table 7, page 128; Figure 17, page 129). The simplest explanation for this is that at high degrees of substitution the haptenic groups were being superimposed upon native determinants of the carrier and were thereby diminishing the immunogenicity of the carrier. A similar situation has been reported by Parish (1971), who described how monomeric flagellin which had been heavily acetoacetylated was shown to be apparently non-immunogenic when injected into rats. The difference in the response of the chicken to lightly and heavily haptenated HSA could therefore reflect a paradoxical situation: a sparsely substituted carrier carried insufficient hapten to promote a response but was itself recognised well enough, whereas at high levels of substitution the carrier was encumbered by hapten groups, became increasingly non-immunogenic, and was not recognised by "helper" T cells.

It has been shown in mammals (Rajewsky et al., 1968; Katz et al., 1970) and more recently in birds (Sarvas et al., 1974) that pre-immunisation with a small quantity of the carrier alone caused an enhancement of the primary anti-hapten antibody response upon subsequent immunisation with hapten conjugated to the homologous carrier. The rationale for this observation was that pre-immunisation

with a small amount of the carrier stimulated the appropriate population of "helper" T cells into a state of preparation and proliferation prior to subsequent challenge with haptenated carrier. However, chickens pre-immunised with 500 µg HSA 7 days before immunisation with either 100 µg or 10 mg HSA failed to produce significantly greater amounts of anti-DNP antibodies than birds which had not been pre-immunised. In fact, any critical assessment of carrier pre-immunisation was impeded by the finding that only those birds immunised with a large dose (10 mg) of DNP-HSA produced detectable amounts of anti-DNP and indeed in these birds there was no apparent enhancement of anti-DNP production as a result of pre-immunisation (Table 8, page 132). All the birds responded well to the carrier and chickens which were pre-immunised with HSA produced correspondingly greater amounts of anti-HSA antibodies. It has recently been reported by Sarvas et al. (1974) that a large pre-immunising dose (e.g. 200 to 2000 µg BSA or HGG) of carrier actually caused specific inhibition of the subsequent antibody response to the hapten - perhaps because anti-carrier antibody was inhibiting cell co-operation. Thus in the experiment described on page 130, in which experimental birds were pre-immunised with a 500 µg dose of HSA and challenged with DNP-HSA 7 days later (which would have coincided with the attainment of maximum levels of anti-HSA in the circulation of these pre-immunised birds), the amount of anti-HSA present 7 days after pre-immunisation might well have negated any advantage of alerting the carrier-specific "helper" cell population.

Different ways of presenting the DNP hapten (e.g. using

various types of carrier, different epitope densities and pre-immunisation with carrier alone) seemed to have little effect upon the facility of chickens to produce anti-DNP antibodies, and the poor anti-DNP responses made it difficult to evaluate these variations in the antigenic stimulus. A number of years ago it was reported by Wolfe and Dilks (1948) that older birds produced more antibody than did younger birds and they suggested that some of the immunological faculties of chickens were not fully developed until about 5 weeks of age. In addition, certain characteristics of the antibody response in chickens immunised with HSA in FCA have also been shown to be age-dependent (Henderson, 1975). In foetal and neonatal mice the heterogeneity of anti-hapten antibody affinities was apparently much less than in adult mice (Goidl and Siskind, 1974) - again suggesting that certain parameters of the antibody response were age-dependent. In the experiments in which the effects of epitope density, choice of carrier and pre-immunisation were investigated, the birds used were never older than 8 weeks - both because of the availability of young birds and because chickens aged 6 to 8 weeks were known to respond consistently well to immunisation with a protein antigen (e.g. HSA), there was no precedent for excluding them. However, it was found that immunisation of 15-week old adult birds with 5 mg DNP-HSA injected intravenously elicited not only a significant anti-DNP response (Table 9, page 134; Figure 19, page 136) where a similar immunisation schedule in younger birds had proved unproductive, but a strong anti-HSA response was also detected (Table 9, page 134; Figure 18, page 135). Certainly, most previous reports of anti-hapten antibody biosynthesis have involved immunisation of

adult birds (e.g. Orlans, 1968; Yamaga and Benedict, 1975b), although young inbred birds (4 - 10 weeks) have been used for producing anti-NIP (4-hydroxy-3-iodo-5-nitrophenylacetyl) antibodies (Sarvas et al., 1974). The results reported here therefore strongly suggested that the age of the birds was important in determining their responsiveness to dinitrophenylated antigens.

The kinetics of the anti-DNP response in 15-week old chickens are given in Table 9 (page 134) and shown in Figure 19 (page 136), and the pattern of this response may be compared with that of the anti-HSA response in the same birds (Table 9; Figure 18, page 135). There was virtually no anti-DNP activity detected in serum samples 3 days after immunisation, but from then onwards there was a steady increase in serum antibody levels. These antibody levels reached maximum values about 7 days after immunisation and subsequently decreased - although the decline of anti-DNP antibody levels was not as patently exponential as the decline of anti-HSA antibody levels. The pattern of the kinetics of anti-DNP biosynthesis was therefore broadly similar to that for anti-HSA production, implying that the previous discussion about the sequence and timing of events prior to and during secretion of anti-HSA antibody molecules was also tenable for the anti-DNP response. It is worth noting that the specificity of the assay system for anti-DNP was confirmed by the fact that serum from bird number 891, which had been immunised simply with native HSA, failed to bind the radio-labelled DNP-CSA. A comparison of the anti-HSA and anti-DNP responses just described showed no apparent correlation between the two; this supported the proposal that the carrier was indeed playing a secondary role (e.g. facilitating cell

co-operation) rather than merely providing suitably immunogenic locations for hapten groups. If this latter suggestion were correct, then a positive correlation between the antibody response to the carrier and to the hapten moieties might have been expected.

The importance of the nature of the antigen and how it is presented and the relevance of these factors when considering extrinsic control of antibody biosynthesis, have already been emphasised. Immunological memory is an intrinsic control mechanism which usually results in enhanced antibody biosynthesis when an animal is re-immunised with the same antigen as used for primary immunisation. To explain this phenomenon it has been suggested by Sercarz and Coons (1962) that three different cell types, representing different phases in the differentiation of the immunocompetent cell, may be recognised and they have been annotated as follows; x : virgin lymphocytes which have had no contact with antigen, y : memory cells, z : antibody-forming cells derived from x or y as a result of antigenic stimulation. One attractive possibility is that $x \rightarrow y, y \rightarrow z$ and $x \rightarrow z$ transitions all proceed <u>via</u> a common activated blast cell intermediate. The operation of immunological memory is therefore manifest in a secondary response; in the primary response a relatively small number of x cells specific for the antigen are induced to differentiate and proliferate, but some time (a time of 4 days after injection in the case of chickens was arrived at - see page 115) must elapse before the number of antibody-forming cells has expanded sufficiently to produce detectable serum antibody. By contrast, in the secondary response the individual starts with an expanded population of y cells whose

proliferative response to antigen is more immediately reflected by an increase in serum antibody which subsequently reaches a higher level relative to the primary response because of the greater number of committed cells produced. When the DNP group, attached to a different carrier, was used for a second immunisation in adult birds, an anti-DNP response with the apparent characteristics of an anamnestic response (i.e. a more rapid and intense response) was observed. Thus, re-immunisation with 2 mg DNP-HCN of birds primed with 5 mg DNP-HSA resulted in high anti-DNP levels in all the birds (Table 10, page 139; Figure 20, page 140), the peak value of which was up to ten times the corresponding value recorded at the peak of the primary response. If these secondary responses were genuinely anamnestic (with respect to the hapten), then there was no evidence of the "carrier effect"; i.e. there was no apparent requirement that the hapten be coupled to the same carrier both times for it to elicit a response having the characteristic kinetics of an anamnestic anti-DNP response. This might suggest that carrier-specific "helper" T cells were not involved in promoting the anti-hapten response, the DNP conjugates (DNP-HSA and DNP-HCN respectively) behaving effectively as T-independent antigens. On the other hand, it could be that haemocyanin is a more efficient carrier anyway, and that the supposed anamnestic response was in fact a more intense primary response. There was certainly no indication of tolerance to the DNP hapten in the secondary response, making tolerance an unlikely reason for the unresponsiveness to DNP found in young birds. Some of the birds which were primed with DNP-HSA were challenged with native HSA alone; here again there was a marked increase in anti-HSA

levels in the secondary response compared to those seen in the primary response (Table 10, page 139; Figure 21, page 141) and it was evident that those birds which responded well in the primary response also responded well to a second injection of antigen (i.e. if for these chickens the ABC_{30} obtained 7 days after primary immunisation is compared with the ABC_{30} obtained in the same bird 7 days after secondary immunisation, then the correlation coefficient was 0.998 and the probability of obtaining so high a value by chance was $P = 0.02$). This positive correlation would be consistent with the idea that antibody-forming cells and memory cells were derived from a common activated blast cell intermediate, and that consequently the numbers of antibody-forming cells and of memory cells were both dependent upon the number of precursor cells which were activated by antigen.

Whilst a certain amount of information concerning the control of antibody biosynthesis may be inferred from the kinetics of antibody biosynthesis alone, the average affinity and the overall distribution of affinities, and the variation of these parameters under different experimental conditions, provide more detailed evidence about the cell selection process inasmuch as the characteristics of secreted antibody are very probably analogous to those of receptor molecules. Another reason for investigating the immunogenicity of dinitrophenylated antigens in chickens was therefore to establish an experimental system for accurately measuring antibody affinity in chickens.

An equilibrium dialysis system was devised for measuring the affinity of chicken anti-DNP antibodies for the ligand DNP-ε-

aminocaproic acid which incorporated the following features: (1) A wide range of hapten concentrations (a range from 10^{-3} to 10^{-9} M DNP-EACA) was used, comparable for example to the range employed by Werblin and Siskind (1972a) who were dealing with free hapten concentrations at equilibrium ranging from 10^{-6} to 10^{-13} M. This effectively ensured that each antibody subpopulation would, at some of the hapten concentrations used, be partly and only partly saturated with hapten. Unless this condition is attained for every antibody subpopulation, the affinity measured might not be characteristic of the whole population of antibody molecules. In other words, the antibody population effectively studied might be artificially selected if a narrower range of hapten concentrations was used; this might have happened for example in the experiments of Eisen and Siskind (1964) and Little and Counts (1969), who used free hapten concentrations with less than a one hundred-fold difference between the highest and lowest hapten concentrations. (2) Only whole serum or else a crude globulin fraction was dialysed, thus avoiding possible selective loss of high affinity antibody during purification, as has been shown to occur by Lopatin and Voss (1974b). (3) For calculating the geometric mean intrinsic association constant (i.e. the average affinity) a graph of log [bound hapten] against log [free hapten] was plotted; in theory, the concentration of free hapten required to saturate all antibody combining sites could then be read directly from the plateau of the graph (as described on page 97) such that no independent measurement of antibody concentration was required. Once the concentration of combining sites, $[Ab_t]$, had been found, values for the average affinity (K_o) and the heterogeneity

index (a) could then be determined using the relationships (derivation shown on page 95 et seq.) that: (i) the intercept on the log [b] axis is equal to log $[Ab_t] . K_o^a$ and (ii) the slope of the straight line portion of the graph is equal to a.
The logarithmic scale used for the concentrations of both free and bound hapten prevented clustering of the experimental points at one end of the graph. (4) Single containers (300 ml capacity polyethylene bottles) were used for each different hapten concentration, each containing a large volume (200 ml) of hapten solution; Werblin, Kim, Smith and Siskind (1973) have recommended using large volumes of hapten solution in order to minimise errors due to radioactive contaminants and impurities in the hapten solution.

The equilibrium dialysis data is represented typically by the graphs shown in Figure 13 (page 102). These graphs are different from conventional graphs of such data, examples of which are shown in Figure 29; for instance, Eisen and Siskind (1964) and Little and Counts (1969) plotted log r/(n-r) against log c (where r = moles of hapten bound per mole of antibody, n = maximum number of moles of hapten that can be bound per mole of antibody and c = concentration of free hapten). From the straight line drawn through various values of log r/(n-r) and log c, the parameters a (index of heterogeneity) and K_o (geometric mean intrinsic association constant) were determined using the relationship log r/(n-r) = a log c + a log K_o which is the Sipsian adsorption isotherm. A graph of this sort is shown in Figure 29(A). Another method sometimes used is to construct binding curves as Scatchard plots of r/c against r (where

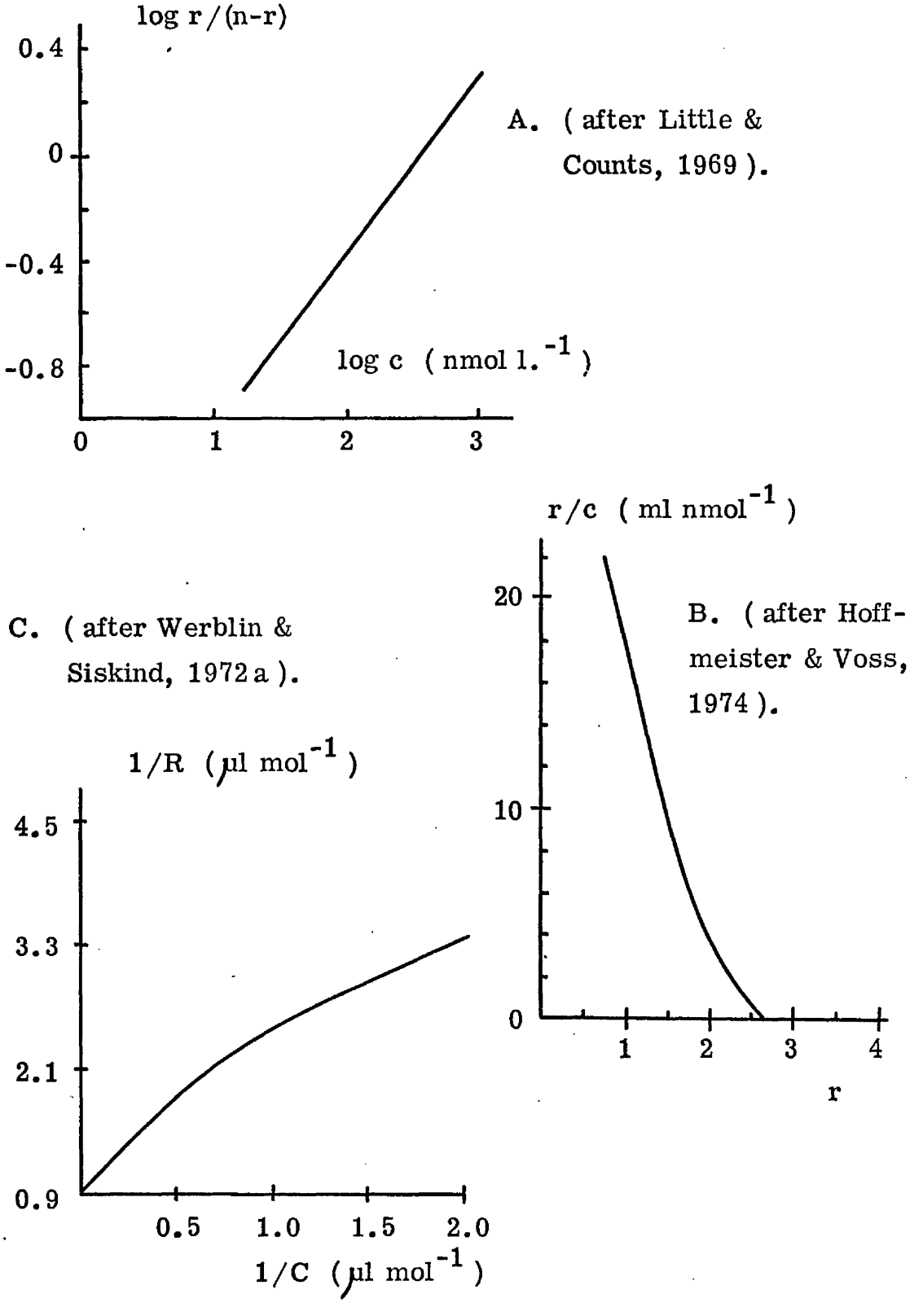


FIG. 29. Representative graphs of equilibrium dialysis data. A. is plotted according to $\log r/(n-r) = a\log K_0 + a\log c$, B. according to $r/c = nK - rK$, and C. according to $1/R = 1/NK.1/C + 1/N$.

r represents moles of ligand bound per mole of antibody at free ligand molar concentration c), as done for example by Lopatin and Voss (1974a) and Hoffmeister and Voss (1974) using the relationship $r/c = nK - rK$ (as before, n is the limiting value for r at infinite c and K is the equilibrium constant). A third method, such as used by Werblin and Siskind (1972a), was to plot 1/R against 1/C according to the equation $1/R = 1/KN.1/C + 1/N$ (where K is the equilibrium constant, R is the concentration of bound antibody sites, N is the total concentration of antibody sites in the reaction system and C is the equilibrium concentration of free hapten). Experimental data plotted according to both these methods are also shown in Figure 29(B & C). For all three methods the equations used are all essentially equivalent and were derived from the law of mass action.

The equilibrium dialysis system and the methods for plotting data and thence determining the affinity and heterogeneity index were given in detail on page 97 et seq., and Figure 13 (page 102) shows two typical plots; the salient features of these two graphs, which serve as an appraisal of the method, were as follows. (1) The portion of the log [b] against log [c] graph at low values of [c] was consistently a good straight line. Values of the heterogeneity index, calculated from the straight line portion using the relationship $\log [b] = a \log [c] + \log ([Ab_t].K_o^a)$, were usually equal to, or just less than 1.0, indicating marked homogeneity of the antibody population. A similar finding has been reported for rat anti-DNP antibodies by Larralde and Faber (1971). (2) From the equations derived for calculating antibody affinity, it was

predicted that a definite, flattened plateau should be obtained at high concentrations of free hapten; in practice (see Figure 13) this was seldom achieved and the concentration of free hapten at which all antibody combining sites were occupied by hapten was indeterminate - often there was no real flattening of the graph (even at free hapten concentrations of 10^{-4} and 10^{-3} M), and in instances where saturation of antibody combining sites with hapten was evident the considerable scatter of experimental points at high values of [c] introduced significant errors in the exact positioning of the "plateau". This predicament was aggravated by the proportionally small amounts of radioactive hapten which could be used at the high concentrations of free hapten, and thus the amount which could bind to antibody was correspondingly small. (3) The values for antibody affinity which were calculated from graphs such as those shown in Figure 13, were consistently low (e.g. K_o between 10^3 and 10^4 litres mole^{-1}), and the question arises whether this sort of affinity can justifiably represent specific binding by antibody; if so, it is difficult to see how antigen would interact with receptors of such low affinity in the first place. Werblin, Kim, Quagliata and Siskind (1973) have operationally defined as specific antibody those combining sites occupied by hapten at a free hapten concentration of 5×10^{-6} M or less. There was therefore an apparent lack of high affinity anti-DNP antibody produced in chickens immunised with a single intravenous injection of either 10 mg or 100 µg DNP_{24}-HSA (Table 11, page 144), and a similar finding has been reported by Yamaga and Benedict (1975a) - a maximum range in $-\Delta G^o$ from 8.28 to 9.19 kcal mole^{-1} (equivalent to

K_o = 8.6 x 10^5 to 3.9 x 10^6 litres $mole^{-1}$) for birds given two injections of DNP-BGG at a variety of doses (e.g. from 20 µg to 20 mg DNP-BGG with an interval between the two injections of 1, 3 or 8 weeks). At the same time, Yamaga and Benedict (1975b) have found that immunisation of chickens with antigen in FCA gave rise to production of higher affinity antibodies, and Hoffmeister and Voss (1974) have given a K_o value of 1.6 x 10^7 litres $mole^{-1}$ for anti-DNP antibodies from chickens which had received three injections of 5 mg DNP-BGG in FCA. In conclusion, it was found that chickens produced anti-DNP antibodies of very low affinity but of considerable homogeneity following an intravenous injection of either 10 mg or 100 µg DNP-HSA. On the evidence of restricted precipitin arcs seen in radio-immunoelectrophoresis, Hoffmeister and Voss (1974) have previously suggested that chicken anti-DNP antibodies seemed to be less heterogeneous than, say, rabbit anti-DNP antibodies. In the light of the findings that adult birds responded well to an immunogen such as DNP-HSA whereas young birds did not, then perhaps in this case the age of the birds (10 weeks) was critical. Alternatively, Mond, Kim and Siskind (1974), in order to explain why rabbits immunised with a single intravenous injection of 15 mg DNP-HCN failed to produce high affinity antibodies, have suggested that at higher antigen doses only low affinity antibody-producing cells were triggered whilst cells of high affinity were tolerised.

Affinity has been used to describe the quality of antibody in terms of its interaction with a simple molecule such as a hapten; since the interaction between antibody and a complex antigen (e.g. HSA) cannot be characterised or defined in quite the same

way (because each antigen is made up of a number of different determinants, probably each of which serves as a target for antibody molecules with specificity for that determinant), certain assumptions have to be made before the law of mass action can be applied to the interaction between antiserum and a complex antigen. For this reason, the quality of binding between antiserum and a complex antigen is described as the avidity of that antiserum - and because of the conditions which have to be made before an avidity value can be calculated, it is only a relative value and cannot be regarded as an accurate thermodynamic expression of binding in the same way as affinity can. Nevertheless, methods for determining antibody avidity have been described, for example Steward and Petty (1972) have reported avidities for mouse anti-HSA antibodies, and a similar method was used here for finding the avidity of chicken anti-HSA antibodies (procedures described in detail on page 86 et seq.).

The effect of a second encounter with antigen upon the antibody response of an individual has already been mentioned and its implications for a selection theory regulated by the operation of immunological memory have been discussed. The same sort of mechanisms might be expected to operate when an individual is given several injections of the same antigen (i.e. primed with antigen and then boosted) - thus the first immunisation would trigger some antigen-sensitive precursor cells to differentiate into antibody-forming cells, others into memory cells; antigen injected subsequently would utilise these memory cells, resulting in a relatively larger number of cells being activated to producing

antibody - and yet more memory cells would be initiated so that a further antigenic stimulus would again result in enhanced antibody production. On the basis of a cell selection theory it might be predicted that repeated injections of the same antigen would cause an animal to produce antibody of progressively higher avidity; i.e. as more antibody was produced in response to successive antigenic challenges, the greater would be the competition between the combining sites on secreted antibody molecules and those on cell surface receptor sites - resulting in selective stimulation of those cell receptors of high avidity. When chickens were immunised with successive 40 μg doses of HSA injected intravenously at weekly intervals (i.e. challenge was made at the presumed peak of antibody biosynthesis resulting from the preceding injection), a consistent pattern in both the antigen-binding capacity and the avidity of antibody was observed (Table 12, page 148). In terms of the absolute amounts of antibody present, there was always a considerable drop in antigen-binding capacities in serum samples taken the day after injection of antigen - indicating that systemic interaction between antigen and antibody had occurred or else that free antigen present in the serum was competing with, and therefore inhibiting, binding of the radio-labelled antigen used in the avidity assay system. However, at least some of each booster injection had obviously reached and stimulated precursor cells, as was evident from the increased levels of circulating antibody observed 6 and 7 days after each challenge (with the noteable exception of those values observed at the end of the second week). The avidity of antibody from each bird in this experiment was significantly higher

at 27 days than it was at 20 days after the initial injection of antigen (e.g. the average avidity at day 20 compared with that at day 27 by a two-tailed t-test gave P = 0.017). There was no discernible pattern of avidity changes for the serum samples taken on 3 days consecutively (i.e. before and after boosting with antigen) in any one week. This observation was consistent with the idea that maturation of antibody affinity or avidity with time after immunisation is a result of selection of antibody-forming precursor cells of progressively higher affinity as antigen concentration becomes less, rather than being due to selective removal of antibody of high affinity (or avidity) from the circulation at the start of the response when antigen is present in copious amounts. The significant increase in avidity at the end of the fourth week could well have been due to the increased amounts of antibody present at the end of three weeks (Table 12, page 148) competing with the antigen-sensitive cell receptors and resulting in cells of high avidity being selectively stimulated to differentiate into antibody-producing cells.

So far, the control of antibody biosynthesis has been related to the immunogenicity of the antigen. However, an antibody response is usually governed by additional factors which may for example modify the immunogenicity of the antigen and influence biosynthesis in this way or else may regulate antibody production once active biosynthesis has started.

THE EFFECTS OF ADJUVANT UPON THE IMMUNOGENICITY OF THE ANTIGEN AND UPON THE BIOSYNTHESIS OF ANTIBODIES

It has been recognised for many years that certain

substances, when administered to an animal in accompaniment with antigen, will cause a potentiation (i.e. will provoke an increase in the amount and/or duration) of antibody biosynthesis to that antigen. For this reason such substances have been termed adjuvants, and their effect upon the control of antibody biosynthesis is obviously of great interest. The theories which have been postulated in the past concerning the mode of action of adjuvants are numerous and each of the different properties of an adjuvant have been implicated at some time or other: thus, adjuvanticity has been attributed, wholly or in part, to a variety of factors, including the depot effect, enhanced activity at cell surfaces, labilisation of lysosomes and increased phagocytosis of the antigen.

A study of the kinetics of antibody biosynthesis in chickens which had been immunised with HSA in FCA enabled certain conclusions to be reached as to how adjuvants may cause modification of antibody biosynthesis. The general pattern (Table 13, page 152; Figure 22, page 153) of the kinetics of anti-HSA antibody production showed that there was an initial phase of biosynthesis analogous to a primary antibody response, and from about 7 days onwards there was a definite decline in antigen-binding capacities for birds immunised with 5 mg HSA, and at least a stabilisation of antibody levels in birds immunised with 100 µg HSA. However, between 3 and 4 weeks following primary immunisation of chickens with either 5 mg or 100 µg HSA in FCA a second phase of antibody production was observed in most of the birds, which persisted for at least 3 weeks. These findings are in agreement with those of French, Stark and White (1970), who

reported similar biphasic responses for birds immunised with 40 µg HSA in FCA. Although antibody levels in the second phase of biosynthesis (that is in birds showing the biphasic response) were somewhat higher than the levels in the same individuals during the initial phase, these increases were certainly not as great as some of those reported by French, Stark and White (1970). However, the second phase certainly lasted much longer than the first phase, and mean ABC_{30} values showed no sign of diminishing up to 48 days after immunisation. The question of how adjuvants affect the control of biosynthesis may be discussed from at least two points of view. The first consideration is that antigen is probably released only slowly from the site of injection, so that small amounts will be present in the circulation and tissues for a considerable time - in contrast to the fate of antigen when it is administered as a single intravenous injection; for example, White, French and Stark (1970) have shown that in chickens a 10 mg dose of HSA was virtually cleared from the circulation by the fifth day after injection. The second factor to bear in mind is the inclusion of mycobacteria in the oil phase of the water-in-oil emulsion. It has been argued by White (1973) that the mycobacteria were responsible for structural disorganisation of germinal centres, and that this in turn disrupted the homeostatic processes whereby specific clones of lymphocytes were segregated into germinal centres: the end result of this being that the homeostasis which regulated the biosynthesis of specific antibody was alleviated. The kinetics of antibody biosynthesis seen here (Figure 22, page 153) were consistent with an initial phase of antibody production, which was regulated to some extent by homeostasis

(e.g. in birds immunised with 100 μg HSA in FCA the mean ABC_{30} levelled off after about the seventh day, rather than showing any tendency to decline whilst in birds immunised with 5 mg HSA in FCA there were more definite signs of homeostasis). Subsequently there was a failure of the homeostatic mechanism, either as a direct result of the action of mycobacteria or else because homeostasis only endures for a finite period of time anyway, and a second prolonged phase of antibody biosynthesis was elicited by small amounts of antigen leaking from the water-in-oil emulsion in the muscle. In some ways the kinetics of the antibody response following immunisation with HSA in FCA, as shown in Figure 22 (page 153), were similar to the kinetics observed in chickens given repeated intravenous injections of HSA at weekly intervals (Table 12, page 148); in the latter experiment, after a refractory phase in the second week, there were marked increases in antibody levels during the third and fourth weeks after the initial injection - suggesting that the homeostasis which regulated the primary response to antigen was no longer effective after this length of time or with repeated antigenic stimulation. Perhaps the same sort of process was happening in birds immunised with antigen in adjuvant. In any case, the duration of the second phase of biosynthesis showed that there had been no repetition of the sharp negative feedback seen in the first phase or in the secondary response provoked by a second intravenous injection of antigen.

There was a significant correlation between the antigen-binding capacity of serum and that of granuloma extract when compared

in samples taken 48 days after immunisation, for birds immunised with 100 µg HSA in FCA (correlation coefficient = 0.989; P = 0.0057) and a fair correlation for birds given 5 mg HSA in FCA (correlation coefficient = 0.788; P = 0.057), and this is shown in Figure 23 (page 156). One plausible explanation for such good correlation is that most, if not all, of the antibody produced at later stages of the second phase in fact comes from the granuloma. This would agree with the findings of French, Stark and White (1970) who reported that there were no genuine anti-HSA-producing plasma cells in the spleen in the second phase of the response in birds immunised with HSA in FCA but that the antibody content of the granuloma at this time (e.g. 54 days) was between four and thirty times greater than that found in any other tissues (including the spleen), and histological examination of the granuloma showed an intense infiltration of plasma cells around the periphery.

Changes in the quality of antibody during the first and second phases of the response following immunisation with antigen in FCA were monitored by the changes in avidity; measurement of avidity of antibody to a protein antigen (HSA) was preferred to measuring affinity of anti-hapten antibodies because of the very low affinity values obtained for anti-DNP antibodies in chickens. It has already been argued that the second phase of the adjuvant-assisted response in chickens could be considered as a secondary response stimulated by antigen still present in an immunogenic form. There remains the question of whether this persistent antigen, which is slowly and continuously released from the antigen depot, stimulates the same clones (in the form of specific memory cells for example) of antibody-

producing cells which accomplished the initial phase of the response or whether it stimulates a different population of cells once the homeostatic regulation has been actively alleviated or has relaxed spontaneously. In the first instance (i.e. stimulation in the second phase of antibody-forming cell precursors analogous to those triggered in the primary phase) an overall increase in antibody avidity between first and second phases (the increase determined by the passage of time and by the exponentially decreasing amounts of antigen released from the water-in-oil emulsion) might be expected. Conversely, if a different and dissimilar population of antibody-producing cells was involved in the second phase of antibody biosynthesis, then an equal or lower avidity in the second phase might be predicted since the higher affinity clones might have been selectively stimulated in the first phase, leaving only the lower affinity clones to respond in the second phase. Such a comparison of avidity for antibody from the first and second phases respectively gave equivocal results (Table 14, page 161). In all cases the avidity of antibody measured at 29 days after immunisation with antigen in either FCA or FIA was higher than the corresponding value after 5 days, and this tendency for the avidity to increase has previously been reported by French, Stark and White (1970). Although it seemed that immunisation of chickens with antigen in Freund's complete or incomplete adjuvant appeared to cause an increase in anti-HSA avidity in the second phase (e.g. at day 29) relative to the first phase of the response (e.g. at day 5), these increases were not statistically significant ($P = 0.13$ and $P = 0.29$ respectively) when compared using a two-tailed t-test. However, these increases,

although not satisfying the statistical criterion $P < 0.05$, did provide some indication, especially when considered along with previous results of French, Stark and White (1970), that time-dependent and dose-dependent increases in avidity had occurred, evidence that a similar cell population (memory cells programmed during the initial phase perhaps) to that which participated in the first phase of the adjuvant-assisted response was also responsible for production of antibodies in the second phase.

Several properties of antibody molecules which help in elucidating control mechanisms have already been mentioned (e.g. the specificity, affinity and heterogeneity of the molecules); another characteristic which has proved useful in this respect is the isotypic heterogeneity of antibody molecules - the immunoglobulin class to which they belong. For example, the proportion of 19S to 7S antibody has been found to vary with the type of antigen (e.g. large antigen molecules made up of regularly repeating subunits tend to elicit production of IgM antibodies predominantly), with the dose of antigen and with time after immunisation (e.g. a general pattern is for early IgM biosynthesis which is subsequently replaced by IgG production). Accordingly, it has been shown by Nielsen (1974) that following immunisation of chickens with SRBC there was an early IgM response which rapidly gave way to biosynthesis of IgG antibody. On page 20 it was argued that this sort of pattern could be explained by preferential stimulation of IgM-producing cells early on by multivalent antigen (because of the advantages of IgM itself being multivalent and therefore having a high net affinity for such antigen)

followed later by activation of IgG-producing cells by partially degraded (oligovalent) antigen - as suggested by Makela et al. (1967). On the one hand this scheme is confounded by reports that both IgM- and IgG-producing cells carry monomeric IgM receptors (Vitetta, Baur and Uhr, 1971), but on the other hand it is compatible with the idea that IgG antibody specifically inhibits production of IgM antibody (e.g. as reported by Finkelstein and Uhr, 1964).

A comparison of the relative amounts of 19S and 7S anti-HSA antibody in the primary, secondary and adjuvant-assisted antibody responses in chickens was made (see Table 15 a & b, page 163, and Figure 24, page 164). In the primary response following immunisation with 5 mg HSA injected intravenously, 7S antibody constituted 71% of the total anti-HSA after 7 days, whereas at the 7-day peak of a secondary response following challenge with 2 mg HSA injected intravenously just over 80% of the total was 7S. There is a considerable discrepancy between these values and those reported by Pierce, Chubb and Long (1966); these authors found that antibody obtained from chickens 7 days after immunisation with BSA (100 mg per kg body weight injected intraperitoneally) consisted of only 33% IgG (67% IgM) as measured by passive haemagglutination with antibody fractions separated by sucrose density gradient ultracentrifugation. Furthermore, Pierce, Chubb and Long (1966) also reported that antiserum obtained from these same birds 7 days after secondary immunisation with 10 mg BSA injected ip contained 45% IgG (55% IgM) anti-BSA. More recently, Yamaga and Benedict (1975a) have shown that anti-DNP antisera which were obtained after two injections of

DNP-BGG (2 mg iv) and then fractionated by gel filtration, consisted of between 37% and 68% IgM when the two injections were given one week apart, and between 29% and 35% IgM when injections were given three weeks apart; for antigen doses other than 2 mg (e.g. 20 µg, 200 µg or 20 mg DNP-BGG) the antibody response was made up entirely of IgG antibodies. The distribution of antibody classes in antiserum samples obtained from birds immunised with 5 mg HSA in FCA (given in Table 15a, page 163) was 63% 7S anti-HSA (37% 19S) in the first phase (day 7) rising to between 80.5% (day 28) and 83% (day 34) in the second phase. Thus the proportion of 7S to 19S anti-HSA in the second phase of the adjuvant-assisted response was very similar to that seen in a genuine anamnestic response. This would fit in with the idea that in the secondary response (and by analogy, in the second phase of the adjuvant-assisted response), memory cells sensitized during the first encounter with antigen were promoting biosynthesis but with the omission of much of the preliminary IgM production seen in the primary response. Perhaps the switch from IgM to IgG antibody production which occurred in the primary response had been maintained in the memory cells.

The modifications in antibody biosynthesis in chickens resulting from administration of adjuvants may therefore be partly attributed to two important properties of the adjuvant mixture: firstly the dispersal of antigen in the aqueous phase of a water-in-oil emulsion and secondly, the presence of mycobacteria - which appears to inhibit homeostasis in chickens (White, 1973) and might anyway be expected to exert some non-specific stimulatory effect (at

least on macrophages and possibly on lymphocytes as well). The operation (and break-down) of homeostatic regulation has been investigated in a different experimental system by Nielsen (1974) and White and Nielsen (1975) who demonstrated that concommittant immunisation of chickens each with 10^{10} sheep erythrocytes and 10^{9} heat-killed Salmonella adelaide cells gave rise to an oscillatory pattern of anti-SRBC antibody biosynthesis which was predominantly 19S antibody and showed at least three successive peaks, 7 to 9, 15 to 17 and 25 to 29 days after injection. It could be argued that the oscillation was due to incomplete homeostasis - which in turn might have been brought about by the S. adelaide or could be attributed to a lack of 7S antibody production allied to the fact that 19S antibody had only a limited capacity for inhibiting further biosynthesis. As with the biphasic response observed in chickens immunised with antigen in FCA, comparison of antibody avidities at successive peaks of biosynthesis should provide some indications of the cellular events involved in the oscillation. With this aim in mind, an attempt was made to elicit cyclical production of antibody to HSA (a T-dependent antigen) by immunising chickens with HSA adsorbed onto SRBC (10^{10} HSA-SRBC per bird) injected together with 250 µg lipopolysaccharide from E. coli. In fact there was no evidence of cyclical biosynthesis of anti-HSA (Table 16, page 168); however, there was an appreciable anti-HSA response - which at least indicated that HSA adsorbed onto SRBC (aided perhaps by the adjuvant action of the lipopolysaccharide) was a potent immunogen. Although the exact quantity of HSA adsorbed onto the sheep erythrocytes was not known, Wolberg, Liu and Adler (1969) have estimated that the maximum amount

of BSA which could be adsorbed onto 6×10^8 tanned sheep erythrocytes was 4 µg; on this basis it would be expected that 10^{10} sheep erythrocytes (i.e. the number of SRBC injected into each bird) would be coated with a total amount of HSA equivalent to not more than 67 µg. It was interesting to note that the decline of anti-HSA antibody levels once the peak had been reached about 7 days after immunisation was much slower than in birds immunised with HSA in saline, and this effect was apparently independent of the inclusion of bacterial lipopolysaccharide. From the experimental results given in Table 16 (page 168) it was noted that the sex of a bird had a direct bearing upon the amount of anti-HSA antibody present in the serum of that bird (e.g. typified by comparing peak ABC_{30} values 6 days after immunisation). The male birds responded significantly better than the female birds of the same age and strain - e.g. ABC_{30} at 6 days ranged from 58.7 to 84.1 µg HSA ml^{-1} for male birds and from 7.9 to 26.8 µg HSA ml^{-1} for females, and comparing these values by a two-tailed t-test gave $P < 0.001$. Although the female birds were slightly smaller in size than the males, it was unlikely that this made much difference to the dosage of antigen (in terms of mg per kg body weight). There remains the possibility that the genes controlling the antibody response are sex-linked or that certain aspects of biosynthesis are subject to some sort of hormonal regulation.

REGULATION OF ANTIBODY BIOSYNTHESIS

The homeostatic regulation of antibody biosynthesis has already been referred to several times; for example, by studying the

kinetics of production of anti-HSA antibody (Figure 15, page 119) and of anti-DNP antibody (Figure 19, page 136) it was obvious that serum antibody levels started to fall about 7 days after immunisation - which could have been due either to the removal of antigen and lack of further stimulation, or else to active inhibition of biosynthesis. The latter hypothesis, i.e. active inhibition,is strongly supported by the observation of a refractory phase in birds given a second injection of antigen 7 days after primary immunisation (Table 12, page 148) and in chickens immunised with antigen in Freund's complete adjuvant (Table 13, page 152). These findings suggested that antibody biosynthesis was being actively regulated and it has been proposed that this takes the form of negative feedback inhibition by circulating antibody. A similar mechanism has been implied for regulation of antibody responses in mammals (Uhr and Möller, 1968). To investigate this proposed mechanism in chickens it was considered justifiable to use passive immunisation with specific antibody as a means of trying to simulate this homeostatic regulation in vivo.

Passive anti-HSA antibody administered 24 hours before active immunisation was found to cause an appreciable degree of immunosuppression (Table 17, page 171; Figure 25, page 172). By comparison, the direct effect of passive antibody injected 48 hours after active immunisation was difficult to assess because antibody in the circulation (e.g. at 7 days) could be attributed partly to active synthesis and partly to survival of the passively administered antibody. It was possible to calculate from the observed rate of antibody decay (between 7 and 10 days) how much passive antibody was likely to survive for 7 days, and to derive by subtraction an adjusted mean ABC_{30} at 7

days which represented the amount of antibody synthesised by the birds themselves (e.g. adjusted mean ABC_{30} = 2.5 µg HSA ml^{-1}). There was, however, no significant difference between this value and the mean ABC_{30} at 7 days for the control group of chickens (which received no passive antibody). On the evidence of this rough calculation passive anti-HSA given 48 hours after active immunisation appeared to be relatively ineffective. Probably it was given too late to have any significant effect on the course of biosynthesis. The timing of events which follow administration of antigen and precede the appearance of large amounts of antibody in the serum has already been discussed. Thus, after 48 hours it might be expected that receptors on progenitor cells had already been triggered and activation of these cells had been induced but that the processes of cellular differentiation and proliferation prior to secretion of specific antibody on a large scale were not fully completed. In this situation passive antibody given 48 hours after antigen might be too late to have much influence on the clonal expansion of activated cells. This argument is reinforced by the work of White, Henderson, Eslami and Nielsen (1975) who have described the localisation of a protein antigen in the chicken spleen: one of the first events was the departure of antigen, complexed with antibody, from the blood into the white pulp of the spleen - and this process took place from about 30 hours after immunisation, indicating that some antibody at least had been produced by this time.

The amount of passive antibody required to achieve inhibition is another important consideration in elucidating the mechanisms of

antibody-mediated immunosuppression and of negative feedback inhibition. From the kinetics of antibody biosynthesis it was noticeable that antibody levels invariably declined once a peak had been reached at about 7 days after immunisation with a single intravenous injection of antigen, and this was independent of the amount of antibody in the serum (e.g. Table 4, page 118). Presumably the amount of passive antibody required for successful immunosuppression is indicative of whether antigen is simply being masked by antibody molecules or whether receptor-bearing cells are actually being incapacitated. Such a proposal would require that large amounts of passive antibody were necessary to physically neutralise the antigen, whilst much smaller amounts might suffice for suppression of antibody-producing cells. The effect of diluting passive antibody upon its ability to suppress the antibody response to a given amount of antigen showed that the degree of immunosuppression was not related in any simple manner to the dose of passive antibody, and significant immunosuppression was achieved with undiluted antibody as well as with antibody diluted 1/64 (Table 18, page 176; Figure 26, page 177). In fact, marked immunosuppression was seen both at low and high concentrations of passive antibody, whereas at an intermediate dose (e.g. passive antibody diluted 1/16) there was little evidence of immunosuppression (Figure 26, page 177). In terms of the tripartite model proposed by Chan and Sinclair (1971) it could be argued that an intermediate amount of passive antibody was insufficient to mask all the antigenic determinants, but conversely, failed to leave enough free determinants on antigen-antibody complexes for recognition by antibody-like receptors on B cells - thereby preventing inhibition via

the Fc portions of antibody molecules in the immune complex from taking place.

It has been shown by Walker and Siskind (1968) that the degree of antibody-mediated immunosuppression was directly related to the affinity of the passive antibody given to an animal. It has already been postulated and shown (Table 14, page 161) that antibody taken from the second phase of the anti-HSA response in chickens immunised with HSA in FCA was probably more avid than antibody from the first phase; French, Stark and White (1970) have reported a similar result. Considering these two hypotheses together, it might be predicted that antibody from the second (high avidity) phase of the FCA-mediated response (e.g. antibody extracted from the adjuvant granuloma) to be more immunosuppressive than an equivalent amount of serum antibody obtained at the peak of a primary response. This idea was tested by passively immunising two groups of chickens with one or other of either granuloma extract or serum antibody from the peak of a primary response. However, there was no evidence of antibody-mediated immunosuppression with either preparation (Table 19, page 180); perhaps this was due to the amount of passive antibody used, i.e. birds received 1 ml of either primary response antibody (ABC_{30} = 10.3 µg HSA ml^{-1}) or granuloma extract (ABC_{30} = 11.4 µg HSA ml^{-1}). These amounts of antibody were considerably less than the quantities used in the investigation into the timing of passive immunisation - where birds received 1 ml of passive anti-HSA (ABC_{30} = 126 µg HSA ml^{-1}), and would not have neutralised the antigen (250 µg HSA) to any great extent.

The homeostatic regulation which restricts antibody biosynthesis in chickens is an antigen-specific mechanism; similar specificity has been demonstrated for immunosuppression mediated by passive antibody, although there have been conflicting reports on whether passive antibody directed against a certain determinant is capable of inhibiting the antibody response to the whole antigen or just to the determinant in question. If antibody is inhibiting biosynthesis by suppressing the antibody-forming cells it would be logical to postulate that antibody directed against one determinant specificity (say for example against a hapten) might also suppress production of antibody to neighbouring determinants (e.g. to the carrier for that hapten). On the other hand, a simple masking effect by passive antibody might be expected to block only those determinants whose specificity was complementary to that of the antibody. The precise specificity of passive antibody, whether it suppresses the response to its own complementary determinant alone or else to the entire antigen of which that determinant is part, can therefore be considered to reflect the mechanism by which the passive antibody is acting. The effect of anti-hapten and anti-carrier antibodies upon the antibody response in chickens to a hapten-carrier conjugate was studied to investigate this proposal. The results for this study (Table 20, page 183) were equivocal insofar as there was too much variation within the experimental groups to enable any meaningful comparison of groups as a whole; none of the differences which did occur were any larger than might be expected to arise by chance, as judged by a two-tailed t-test. In similar experiments carried out in mammals, Brody, Walker and Siskind (1967) found that

when two haptens were coupled to the same carrier, and this antigen was used for immunisation, then antibody to one hapten suppressed the response only to that hapten. Conversely, Henney and Ishizaka (1970) have demonstrated that both anti-hapten and anti-carrier responses could be inhibited by passive antibody to either the hapten or to the carrier alone.

For the antibody response elicited in chickens by a single intravenous injection of antigen, whether that antigen is soluble protein, hapten linked to a carrier or else particulate matter, the kinetics of biosynthesis have shown that the decline of the primary response invariably started between 6 and 9 days after immunisation. This in itself contradicted the idea that removal of antigen presaged the decline in antibody biosynthesis since it might be expected that different types of antigen were catabolised at different rates; rather it suggested that the same homeostatic mechanism was operational in the case of each different antigen, and this was independent of the type of antigen but dependent upon the presence of antibody (or antigen-antibody complexes). Accordingly, removal of specific antibody from the circulation would be expected to postpone the onset of homeostasis, and this has been demonstrated by plasmapheresis of immune rabbits (Graf and Uhr, 1969; Bystryn, Graf and Uhr, 1970). Another way of achieving the same objective was attempted in chickens, namely to give repeated injections of antigen during the course of primary antibody biosynthesis which would ensure that all the antibody produced by the birds was immediately complexed with antigen. This would at least help to decide whether the feedback is

mediated by free antibody or by immune complexes. In the case of the anti-HSA response it was found that supplementary injections of HSA, given for five consecutive days beginning three days after the initial immunisation, caused both an increase in the levels of anti-HSA detected and apparently caused a few days' delay in the attainment of peak antibody levels in the serum (Table 21, page 186; Figure 27, page 187). However, even allowing for the amount of antibody removed from the circulation by the supplementary injections of antigen, it was still true that peak antibody levels were not reached before the tenth day in three out of four birds. It would therefore appear that the supplementary HSA injections were able to delay the action of the homeostatic process. This rather weighs against the proposal of Chan and Sinclair (1971) that inhibition is mediated by antigen-antibody complexes in antigen excess.

In contrast, supplementary injections of DNP as a divalent hapten had no demonstrable effect on the homeostasis of either the anti-DNP or the anti-HSA response. White (1973) has proposed that homeostasis is achieved by the localisation of antigen-antibody complexes onto the surfaces of dendritic cells which leads subsequently to the segregation into germinal centres of antibody-forming precursor cells which are circulating in the white pulp of the spleen. This implies that homeostasis could only be postponed until all the antibody-producing B cells capable of responding have been segregated into germinal centres. Once this happens, the bird becomes refractory to further antigenic stimulation (as was noticed, for example, in chickens which were immunised at weekly intervals and which showed evidence of a refractory phase during the second week after primary

injection - see Table 12, page 148).

Although feedback homeostasis in chickens has been attributed to antigen-specific antibody, it was recently shown by Fitch (1974) that anti-idiotype autoantibody was produced during the later stages of an antibody response in rabbits which was specifically reactive to those idiotypes produced during the early stages, and it was suggested that this autoantibody may regulate the response. To test for a similar phenomenon in chickens, chicken anti-HSA antisera taken at various times during the course of an antibody response were screened for anti-idiotype activity by testing each antiserum against the others from the same individual in a double diffusion system. This admittedly simple test gave no indication of the presence of anti-idiotype activity in any of the antisera. This further supports the concept that homeostasis in the chicken is brought about by antibody specific for the antigen.

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