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Immunological Studies in the Domestic Fowl with particular reference to immunological unresponsiveness

A B S T R A C T

Immunological unresponsiveness to soluble protein antigens has been studied in the domestic fowl using bovine gamma globulin (BGG) and bovine serum albumin (BSA).

(SECTION C)

Because the serological behaviour of chicken precipitating antibody differs markedly from that of typical mammalian antibody, it was necessary to undertake a preliminary investigation of the chicken anti-BSA and anti-BGG precipitin systems. Both systems were studied by carrying out quantitative precipitin tests and analysing the precipitates for total N and antigen N. Use of ^{131}I-trace-labelled antigen in these tests made it possible to extend the analyses into the region of considerable antigen excess.

(i) Neither system exhibited a well defined peak of maximal precipitation but produced a broad plateau over that region.

(ii) No true equivalence point could be established for in moderate antibody excess precipitation of antigen was incomplete.

(iii) The peculiarities of chicken antisera raise analytical difficulties that are most easily overcome by the use of labelled reagents

(iv) A rapid method for the determination of the precipitin content of chicken antisera, using ^{131}I-trace-labelled antigen, is described. It depends upon determination of the percentage of added antigen precipitated at the point of maximal precipitation.

(SECTION D)

Although the chicken is a very good producer of precipitins to native serum proteins this immunological ability does not seem to extend to chemically altered antigens. In two experiments in which chickens were immunised with 3 different forms of chemically altered BSA only feeble antibody responses were elicited even after 3 intravenous injections.

(SECTION E)

To study immunological unresponsiveness to soluble protein antigens both embryos and newly hatched chicks were injected with varying amounts of BGG or BSA. Only with difficulty was unresponsiveness induced, the best results being obtained by giving repeated doses of large amounts (100 mg) of antigen during the first few weeks of life. The results verify the general observations that the duration of unresponsiveness is finite and related to the amount of antigen given in early life to induce it, and that maintenance of the unresponsive state depends upon persistence of antigen.

The greater difficulty experienced in making chickens unresponsive to BSA compared to the rabbit and mouse appears to be directly related to the very rapid elimination of BSA by the chicken.

The need for further quantitative studies of unresponsiveness is stressed and a method that is considered suitable for the measurement of a standard degree of unresponsiveness is proposed. The chicken is suggested as a useful experimental animal for the type of quantitative study envisaged.

(SECTION F)

The effect of a cytotoxic drug, 6-mercaptopurine on precipitin production in the adult fowl, was investigated. This purine analogue has been reported capable of causing a complete specific suppression of antibody response to BSA in the rabbit if given as daily doses during period of primary immunisation. Even when the drug was administered at a level three times as great as that known to be effective in rabbits the antibody production of treated birds was equal to that of untreated contols. The failure of 6-MP to suppress antibody formation in the chicken may be due to the particularly vigorous production of precipitins by this species or to a more rapid catabolism and excretion of the drug by the fowl.

IMMUNOLOGICAL STUDIES

in the

DOMESTIC FOWL

with particular reference to
immunological unresponsiveness

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SECTION A

GENERAL INTRODUCTION

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1. The immunological inertness of the young animal

It is one of the natural phenomena of immunology that the newborn and young of most animal species do not possess a well developed immunological faculty and that following stimulation with a material known to be antigenic for the mature individual, they do not produce antibodies or, at best, give only a feeble response (Freund, 1930; Wolfe & Dilks, 1948; Burnet & Fenner, 1949; Edsall, 1953). As the young animal matures so also does its ability to give an antibody response. The time taken to achieve full immunological reactivity varies with the species of animal and with the criteria used to assess the magnitude of the immune response.

A similar non-reactivity is also observed in the embryo. For example, Grasset (1929), Weinberg & Guelin (1936) and Burnet (1941), were unable to demonstrate an immune response in young chickens that had been "immunised" during embryonic life, and experimental embryologists have for years been exchanging tissue grafts between embryos with relative impunity (Danforth & Foster, 1929; Eastlick, 1941; Rawles, 1948). Such tissue transplants generally failed to excite the "incompatability" reaction observed in mature animals and frequently survived for considerable periods of time, whereas in adult animals they would have died within 10 - 14 days.

Thus, it was accepted that foetal mammals, chick embryos and young animals generally were not capable of a normal immune response. Their complete or partial indifference to antigen was regarded as being a consequence of the immaturity of the antibody-forming system. Apart from these purely qualitative observations there was a

dearth of information about the sequential development of antibody forming capacities in different species.

2. Natural erythrocyte chimerism

The first indication that the mere indifference of the young animal to antigen was only part of the story came from the observations of workers who were studying the inheritance of blood groups in cattle (Owen, 1945; Owen, Davis & Morgan, 1946). They noted that twin cattle showed identical blood groups more often than would have been expected from the estimated frequency of identical twinning. It had long been known that in bovine twins, anastomosis of placental blood vessels is a common occurrence, so it would not have been unreasonable to expect some degree of red blood cell admixture in young calves, due to an embryonic exchange of circulating red cells, always assuming that the young animals were incapable of destroying the foreign cells by the normal immunological mechanisms. In such circumstances the duality of blood groupings would have disappeared in the first few weeks of life with the expiry of the natural life span of the red cell or due to the development of an immune response by the host. However, the startling fact revealed by Owen and colleagues was that even when 2 years old these twin cattle still possessed two genetically distinct types of red blood cells, usually but not always, in approximately 50-50 amounts. The existence of such quantitatively stable erythrocyte mixtures in adult animals made it evident that the effective exchange between the embryos had been of potentially haemopoietic cells that had undergone a natural transplantation.

3. The 'adaptive enzyme' theory of antibody formation and the 'self-marker' concept.

No general significance was read into Owen's

observations until Burnet and Fenner in 1949 propounded their 'adaptive enzyme' theory of antibody production. According to their interpretation of the immune response, cells of the reticulo-endothelial-system were held to possess appropriate enzymic machinery for the breakdown and disposal of effete and damaged body cells as part of their normal function. Foreign organic matter was dealt with by the same cells but a change in the specificity of the enzymic system was required before removal of the foreign matter could be effected. To meet this requirement the enzymes had to adapt themselves to their new substrates. Serum-borne antibodies of the conventional type were considered to be replicas of the specifically modified intracellular enzyme system carrying the antigen-adsorptive pattern, but lacking enzymic activity, so that they could combine with, but not degrade, the antigen.

To explain why an animal does not produce antibodies to its own tissue antigens Burnet and Fenner introduced a 'self-marker' concept that indirectly provided the stimulus for much of the outstanding research on 'immunological tolerance'. They postulated the existence of a small number of self-marker components in expendable body cells by which they could be recognised as autologous by the body's macrophage system. Recognition of self-pattern was possible only during embryonic or immediately post-embroyonic stages and recognition resulted in the elaboration of an intracellular enzyme system for dealing with the particular pattern recognised. The freshly established enzymic pattern became part of the inheritable structure of the cell and was transmitted to successive generations. Once the animal assumed an independent existence the ability to establish further intracellular enzyme systems terminated and from then onwards, under normal circumstances, all new matter was regarded as foreign and was dealt with by adaptation of existing enzyme systems and eventual antibody formation.

In formulating the part of their theory that dealt with the limitation of the self-recognition period to embryonic life, Burnet and Fenner had been much influenced by the work of Owen relating to natural erythrocyte chimeras in dizygotic cattle twins, and by Traub's studies of lymphocytic choriomeningitis in mice, particularly his observations that young mice infected with virus in utero, and carrying the infection into adulthood, possessed neither neutralising nor complement-fixing antibodies (Traub, 1939). On the basis of these two sets of observations and as an integral part of their general hypothesis, Burnet and Fenner postulated

(I) that if genetically foreign cells were introduced into an embryo, and became established, they would not evoke an antibody response when the animal became immunologically mature,

(II) that generalised intra-uterine infection of an embryo with a pathogenic micro-organism would preclude the adult animal from forming antibody to the same organism.

These two theoretical ideas were well suited for testing the validity of the hypothesis by direct experiment. Concerning the consequences of prenatal infection there already existed certain relevant evidence. Grasset (1929) had injected diphtheria toxoid into developing eggs but had failed to confer immunity in this way. Burnet himself, in 1941, working with influenza virus, had inoculated living virus into chick embryos and been unable to demonstrate antibodies in the hatched chicks. Non-lethal infection of chick embryos with yellow fever virus resulted in antibody formation only in those chicks which retained circulating virus at the time of hatching (Fox & Laemmert, 1947). Comparable results were obtained with influenza

virus in the work of Burnet and Stone (see Burnet & Fenner 1949: Burnet, Stone & Edney, 1950) but when hatched chicks were again given an injection of virus they responded normally. There was a similar absence of effect on subsequent immune response when non-infectious antigens, bacteriophage and mammalian red cells, were used for embryo injections. Whereas these findings did not fully substantiate their theory of the consequences of embryonic exposure to antigen, Burnet and Fenner were of the opinion that their main implication was that modification of immunological reactivity could not be reproduced by the mere presence of antigen in the embryo.

4. Experimental erythrocyte chimerism

Besides Owen's description of the bovine red blood cell chimeras, there came to light rarer instances of the same natural phenomenon in two other species, the sheep (Stormont, Weir and Lane, 1953), and man (Dunsford, Bowley, Hutchison, Thompson, Sanger and Race, 1953). In addition, a number of successful attempts were made by Owen and other workers to reproduce experimentally in laboratory animals the type of erythrocyte mosaicism seen in nature.

Owen (see Owen, 1956) placed pairs of young rats in parabiosis and the partners developed intermixtures of circulating red cells within 40 hours, though the exchanged cells did not survive long once the partners were separated. On the other hand the intravenous injection of rat foetuses with homologous foetal liver and spleen cells established an erythrocyte mosaicism that persisted for at least several months. Parabiosis was also the tool adopted by the Czech worker, Hasek, who devised a method for fusing the chorioallantoic vessels of avian embryos, the parabionts separating at the time of hatching. Using this technique

between birds of the same species persistent red cell mixtures were obtained (Hasek, 1953). A similar observation was made by Billingham, Brent and Medawar (1956a) after experimental parabiosis in chickens. Too, it was found that even after chimerism had disappeared there often remained an inhibition of the formation of isoagglutinins (Hasek, 1953). Interspecific parabiosis of avian embryos did not result in erythrocyte chimerism with the exception of one case reported by Hasek (1956) in which a chicken retained significant amounts of turkey red cells up to the 8th week after hatching.

Other investigators, working with heterologous red cells, used the less elaborate technique of intravenous injection of embryos or neonatal animals. While their experiments never resulted in chimerism they were often able to suppress agglutinin formation. Simonsen managed to depress the immune response of chickens to human 'O' cells (1956) and to turkey but not to goose erythrocytes (1955) by giving the corresponding cells before hatching. Such suppression of response was evident when the chicks were 6 weeks old but had disappeared by the time they were 12 weeks of age. Owen (1956) found a similar tendency for a depression and delay of specific antibody formation in chickens, rats, and rabbits that had been given injections of human 'O' blood within a day or two of hatching and birth. Working with a variety of avian species and using different interspecific combinations, Hasek (1956) was able to demonstrate a suppression of agglutinin formation only in the case of ducks injected with goose blood. Unlike Simonsen (1955) he did not obtain evidence of suppression of the immune response of chickens to turkey blood. Failure

to demonstrate a depression of response was also recorded by Ambrus and colleagues (Ambrus, J.L., Ambrus, C.M., Johnson, Back, Packman, Chernick & Harrison, 1955) who injected 6-day chick embryos with rabbit red cells, and by Bauer, Peckham and Osler (1956) who gave sheep red cells to rat foetuses on the 15th day of gestation, but these latter workers did obtain a significant reduction of antibody response in rats injected just after birth.

His many experiments with several avian species enabled Hasek to make the following generalisations with respect to the experimental suppression of immunological reactivity to foreign erythrocytes

(I) The greater the taxonomic relationship between donor and recipient species the more readily is suppression achieved.

(II) Embryonic parabiosis causes a much greater degree of suppression than a single intra-embryonic injection.

(III) It is easier to induce suppression in some species than in others.

5. Tolerance in relation to homografts

The various research activities described above were essentially designed to follow up Owen's original description of erythrocyte chimerism in dizygotic cattle twins as a consequence of placental vascular anastomosis (Owen, 1945) and the theoretical concept of immunological tolerance elaborated by Burnet and Fenner (1949). On the basis of the red blood cell studies of Owen (1956) in rats and of Hasek (1953) in chickens it seemed reasonable to assume that tolerance of homologous erythrocytes could be achieved in these species. With heterologous red cell systems on the other hand, experimental evidence did not support the idea

that the introduction of foreign erythrocytes into embryos or young animals could establish a true tolerance to these cells, a hope which had been expressed by Burnet and Fenner (1949), though it did seem reasonably clear that neonatal injection of this type could have conspicuous inhibitory effects on later immune response to the same type of erythrocytes.

The most penetrating and critical analysis of the phenomenon of tolerance came not from such studies with red cell systems but from the field of tissue homografting and that by a rather fortuitous circumstance.

The futility of transplanting tissue grafts (e.g. skin) from one individual of a species to another was well known (cf. Medawar, 1946). Though such a graft might appear to live healthily for a short period, it was inevitably sloughed after 10 - 14 days, the reaction being mediated by an immune response to the individual specific antigens of the donor tissue. Only in the case of identical twins, between which no genetic difference existed, could homografts remain viable. Because of their intimate association with homograft research, Medawar and colleagues were requested to devise a foolproof method of distinguishing monozygotic from dizygotic cattle twins. In attempting to do this they made the startling discovery that most dizygotic cattle twins would accept skin grafts from each other, but would reject skin from a third individual in the usual fashion (Anderson, Billingham, Lampkin and Medawar, 1951). Although it was not possible to distinguish between the two kinds of twins by skin grafting techniques, it was apparent that the failure of homograft rejection by dizygotic cattle twins rested on the same principles as the erythrocyte chimerism

described by Owen, and that an experimental method was available for inducing adult animals to accept tissue homografts by rendering them chimeric with their eventual graft donors. After recourse to experiment this was successfully achieved in mice and chickens by the respective injection of feotuses and embryos with donor tissue cells (Billingham, Brent and Medawar, 1953), the accomplishment marking a significant step forward both in the study of tissue homografting and in investigations relevant to immunological non-reactivity.

Application of this new experimental tool to tissue homograft research focussed considerable attention on the phenomenon of tolerance and produced much of the evidence relating to its essential nature. Outstanding in this respect has been the work of Billingham, Brent and Medawar who were largely responsible for formulating the characteristics of tolerance (see Billingham, Brent and Medawar, 1956b). The term 'immunological tolerance' was originally coined by these workers in the context of tissue transplation immunity; they expressed their definition thus: 'that mammals and birds never develop, or develop only to a limited degree, the power to react immunologically against foreign homologous tissue cells to which they have been exposed sufficiently early in foetal life', and because the phenomenon is the exact inverse of actively acquired immunity they termed it 'actively acquired tolerance' (Billingham, Brent and Medawar, 1953).

Tolerance in relation to tissue grafting is a specialised and technical subject that lies outwith the scope of this thesis, except in so far as the principles of tolerance and its mechanism of action, so carefully analysed by workers in this field, are applicable to related studies with soluble protein antigens. The

realisation of the importance of tolerance to transplantation research, and the application to its various branches of study of methods for the induction of 'actively acquired tolerance' have led to such rapid and widespread developments in this field of biological research that a specialist journal - The Transplantation Bulletin - has been produced to carry the new and valuable information that is coming to light.

6. The characteristics of tolerance

The characteristics of immunological tolerance have been well delineated by Billingham, Brent and Medawar (1956b) and by Medawar (1956) and will be mentioned only briefly here.

(I) The concept of tolerance is not confined to the homograft reaction but is one that is generally applicable in different immunological systems, representing a particular aberration of immunological responses of all kinds.

(II) Tolerance shows true specificity in the accepted sense that an animal may become tolerant of one particular antigen without prejudicing its power to respond normally to any other antigen.

(III) Tolerance represents a central as opposed to peripheral failure of the immune response. The state of tolerance, as shown by persistence of a homograft, can be terminated and the graft rejected if the host animal is given an injection of lymph node cells from a normal adult animal of the same strain. That the passively transferred cells are capable of undertaking an

immune response clearly demonstrates that in a tolerant animal there is neither hindrance of access of antigen to the seat of immunological activity nor any inhibitory effect on the implementation of the immune response.

7. Biological implication of tolerance

Tolerance, being applicable to diverse immunological systems, naturally has many important repercussions and it is appropriate at this point to consider some of its major biological implications. The subject has been well covered by a number of authors (e.g. Billingham, Brent and Medawar, 1956b; Owen, 1957; Chase, 1958) and is only briefly treated here.

A. Mother-foetus immunological relationship: It is more usual to consider the foetus as a potential source of antigen to the mother as, for example, in haemolytic disease of the newborn, but the reverse may apply and the foetus be influenced by antigens of maternal origin. For instance, in humans a Rh-negative foetus can be rendered at least partly tolerant of the Rh antigens derived from its Rh-positive mother, though not to a degree sufficient to alter the incidence of haemolytic disease amongst the children of 'tolerant' Rh-negative mothers. (Billingham, Brent and Medawar, 1956b; Ward, Walsh and Kooptzoof, 1957; Galton, 1960). Experimentally it is possible to induce in non-susceptible mice, a state of tolerance to certain tumours by using the technique of neonatal injection of living cells (see later, p.18) and there is some evidence that a natural tumour homografting of this type can occur in humans as the result of transfer of melanomatous tumour cells from mother to foetus. In the same way the passive transmission

from mother to foetus of foreign antigens of an infectious nature (bacterial antigens, viruses) could have far reaching consequences.

B. Neonatal infections: Many infectious diseases can be contracted at or near birth, and in the case of species whose immunological capacity is still not fully developed at this time neonatal disease could conceivably hamper the ability to resist the same organism in later life (see later, p.19).

C. Auto-immune reactions: Burnet and Fenner (1949) were the first to appreciate that the failure of an animal to react immunologically against its own body constituents is a very important aspect of immunology, and one that requires adequate explanation. Their 'self-marker' concept was an attempt to provide just this. That there are known instances of the body reacting immunologically against its own tissues does not disprove their ideas but provides the exceptions to prove the rule; for the tissues concerned in auto-immune reactions are essentially ones that have formed after the immunological system has stabilised e.g. spermatozoa, and to which no tolerance could have been induced, or the antigens are provided by materials that are normally inaccessible to the centres of the immune response e.g. lens protein, thyroglobulin (see Brent and Medawar 1959).

D. Tissue homografting: The implications of tolerance for this field are obvious. Although so widely explored in laboratory animals there is no reason to doubt that the principle of tolerance applies equally to humans as chimerism does occur naturally in man (Dunsford, Bowley, Hutchison, Thompson, Sanger and Race, 1953). Recent work on the type of 'specific' unresponsiveness, analogous to tolerance,

that can be achieved with soluble antigens has fostered the hope that prolongation of the life of homografts might be brought about by means other than the injection of living tissue cells, and has emphasised the need to isolate and define the tissue antigens responsible for homograft immunity (Medawar 1961).

8. Forms of immunological non-reactivity other than tolerance

The immunological tolerance described in considerable detail by Medawar and his group is not the only form of immunological non-reactivity. There are several descriptions of suppression of the immune response in different immunological systems. Some of these are apparently analogous to immunological tolerance while others seem to possess a different modus operandi. For purposes of comparison it is desirable to describe briefly the various other manifestations of immunological inertness and to comment on their points of resemblance to or difference from immunological tolerance.

(1) Inhibition of drug allergy: One of the first observations of this phenomenon was made by Sulzberger (1929, 1930) when studying the sensitising properties of neoarsphenamine. His guinea pigs could be sensitised by intradermal injections of 150 µg of the drug and would develop a delayed type hypersensitivity, but the response could be prevented if a prior intravenous injection of the order of 6mg was given. In 1946 Chase found that he could prevent, in guinea pigs, the delayed hypersensitivity type of skin contact dermatitis that follows the application of such chemical

compounds as picryl chloride and 2:4-dinitrochlorobenzene, by feeding the compounds to his experimental animals before exposing them to skin contact. It was further shown by Chase (1953, 1955) that the essential failure in the immunological chain of events is a central and specific one. The inhibition of drug allergy therefore closely resembles immunological tolerance for, as Medawar (1956) has pointed out, there is no effective difference between the two states of unresponsiveness although the means by which they are achieved are very different indeed.

(11) Tumour growth enhancement: Strains of mice which are able to resist the development of certain mouse tumours can be rendered at least partially susceptible to these tumours if they are treated with a variety of dead lyophilised tissue preparations in the period immediately preceding transplantation. Thereafter the tumour will enjoy an enhanced growth in the previously non-susceptible mice. This subject has been well reviewed by Snell (1952). While the precise mechanism of this phenomenon is in doubt, it clearly differs from immunological tolerance in that suppression of resistance to tumour growth is achieved by treating young adult mice and not foetuses or neonates. Medawar and associates (Billingham, Brent & Medawar, 1956b) hold the view that tumour enhancement represents a form of afferent inhibition mediated by an inactivation of transplantation antigens issuing from the tumour tissue.

(III) Immunological paralysis: This form of immunological non-reactivity was described and named by Felton in 1949. The injection of mice with very small amounts (0.5 µg) of type-specific pneumococcal polysaccharide served to protect them from later lethal infections of virulent pneumococci of the same type. A much larger dose of polysaccharide (0.5mg) not only failed to protect the mice but thereafter they were not capable of being rendered immune even with small doses of polysaccharide. It was this impairment of response which was termed 'immunological paralysis'. It was found to be specific in nature and of long duration (15 - 18 months)(Felton, Kauffman, Prescott & Ottinger, 1955). Between tolerance and paralysis there lay the important difference that the pneumococcal polysaccharide persisted for a very long time in the tissues, in a form in which it could combine with specific antibody, and indeed there was experimental evidence to show that this was the case, for passively introduced antibody was removed from the serum of paralysed mice much more rapidly than from the serum of normal mice (Dixon, Maurer & Weigle, 1955). The essential nonreactivity of paralysed mice has been ascribed by Stark (1954) and by Medawar (1960) to the constant absorption of antibodies by the vast excess of antigen present in the tissues. In other words an 'efferent inhibition' mechanism appeared to be operative, indicating a difference between tolerance and paralysis. The more recent work of Sercarz and

Coons (1959) using immunofluorescent techniques has shown that paralysed mice are not producing antibody. Thus the inhibition of antibody formation is considered not to be due to the mopping up of antibody by antigen but to involve a more fundamental mechanism. In this case paralysis may not in fact be so very much different from tolerance.

(IV) Protein overloading: Dixon and Maurer (1955) administered extremely large amounts of heterologous serum proteins to adult rabbits and by so doing rendered them temporarily unresponsive to the antigen used, though the animals were capable of a normal antibody response to other protein antigens. The unresponsiveness lasted only as long as the heterologous proteins were detectable in the host (3 - 4 months). Essentially similar findings were reported by Johnson, Watson and Cromartie (1955). Though superficially reminiscent of Felton's immunological paralysis there was no evidence to suggest that the form of unresponsiveness induced by protein overloading resulted from a simple neutralisation of antibody (Dixon and Maurer, 1955), and Sercarz and Coons (1959) were unable to demonstrate antibody in adult mice rendered unresponsive to BSA. Thus this type of unresponsiveness, assuming that it represents a central failure of immune response, would appear to resemble tolerance.

(V) Unresponsiveness induced after whole body irradiation: In the same series of experiments as those on protein overloading of normal adult rabbits Dixon and Maurer (1955) investigated the behaviour of adult rabbits which received similar large doses of heterologous serum protein shortly after exposure to 400r whole body irradiation. Irradiated rabbits remained specifically unresponsive for much longer than normal rabbits given large infusions of foreign protein. The authors considered this might be due to acceptance of the foreign protein as non-antigenic by the host in the immediate post-irradiation period when the virtually depleted immunological system was re-establishing itself. If this were true then clearly there would be a considerable similarity between this type of unresponsiveness and immunological tolerance. In full accord with this finding has been the discovery that lethally irradiated animals will survive if given an injection of foreign bone marrow cells which successfully repopulate the anihilated haemopoietic centres. 'Radiation chimerism' of this type appears to involve principles fully coincident with those of tolerance induced by injection of perinatal animals with living tissue cells (Trentin, 1958).

The preceding five types of immunological inertness have in common the fact that they can be impressed upon animals fully mature in the immunological sense. In this respect they differ from immunological tolerance which holds as one of its essential criteria that it can be established only in immunologically immature animals whether

foetal or neonatal. However, from the point of view that they almost certainly represent central failures of the immune response, both the inhibition of drug allergy and the unresponsiveness induced in irradiated rabbits closely resemble tolerance. Immunological paralysis, originally regarded as a different type of mechanism now appears to be only quantitatively different from tolerance and the same probably holds true for protein overloading of normal adult animals. On the other hand, the phenomenon of enhancement clearly differs from tolerance in this and other respects.

9. Expressions of tolerance in various immunological systems

A. Tumour Transplantation: Mention has already been made of the fact that the concept of immunological tolerance is not confined to the homograft reaction but is generally applicable to a variety of immunological systems, and there are several examples of its expression outwith the homograft field. Most closely related perhaps are such tumour transplantation studies as those of Koprowski and co-workers (Koprowski, Theis & Love, 1956), who were able to get a strain specific C_3H ascites tumour to take in a non-susceptible strain of ICR mice by injecting ICR foetuses with C_3H blood. Successful passage of a mammary carcinoma to resistant strains of mice was also achieved by Aust, Martinez, Bittner & Good (1956), using a similar technique for inducing tolerance, and Simonsen (1955), after injecting chicken blood into 13-day old turkey embryos, was able to infect the hatched birds with Rous chicken sarcoma when they were 8 weeks old, which is well beyond the normal 3 week period of susceptibility.

B. Inhibition of resistance to infectious disease: Especially significant amongst the biological implications of immunological tolerance is its relation to infectious disease. Prenatal and neonatal infections of viral, bacterial and even helminthological origin are very real hazards to the foetus and the newborn animal. Applying the principles to tolerance to such circumstances it can be appreciated that a non-lethal infection in such a very young host could seriously impair the immunological mechanism and render the animal incapable of producing an effective immune response to the same organism in later life. Burnet and Fenner had actually quoted a situation of this type as an example of what they meant by tolerance, for Traub (1939) had found no antibody in adult mice carrying a viral infection originally contracted in utero.

It was inevitable that a number of workers should give their attention to an experimental examination of this aspect of tolerance. In no case was a complete tolerance obtained though there were some instances of a suppression of antibody production. Buxton, (1954) for example, injected killed Salmonella pullorum into developing chick embryos on or before the 15th day of incubation and found that the hatched chicks showed a marked decrease in their capacity to produce demonstrable antibody after oral infection with the same organism. Working with Trichomonas foetus, a protozoan flagellate parasitic in the genital tract of cattle, Kerr and Robertson (1954) observed that the intramuscular injection of T. foetus antigen into very young calves up to 4 weeks of age seriously impaired the subsequent immune response to the same antigen but not to other antigens. New born and 5-day old rabbits given massive doses of heat inactivated Salmonella paratypi B showed a significant inhibition of antibody

formation when challenged at 4 months of age, but no suppression was observed in rabbits that had originally been given only moderate doses of the organism (Sterzl and Trnka, 1957). These workers also obtained similar results in puppies. The experiments of Cohn (1957) produced negative findings. He injected different groups of 14-day old chicken embryos with diptheria toxoid, bacteriophage T2 and pneumococci type II and challenged the chicks when three months old with a mixture of the homologous antigen and another. In no instance did he find any difference in ability to produce antibody between those animals injected as embryos and those not so treated. The negative results of Cohn in the above mentioned experiments were reminiscent of the failure of Burnet et al. (1950) to demonstrate an impairment of the immune response of chickens that had received embryonic injections of influenza virus or bacteriophage.

With respect to infectious agents therefore, there was no clear cut indication that a lasting state of tolerance could be effected by embryonic or neonatal exposure to antigen. There was evidence in certain instances of a significant suppression of later immune response, and in other cases that early experience of antigen did not prejudice later ability to form antibody. More recent work has not helped to clarify matters. Smith and Bridges (1958) attempted to produce unresponsiveness in rabbits to a variety of bacterial antigens with consistently negative results while Lindorfer and Subramanyam (1959) found that rabbits exposed to staphylococcus toxoid early in life had a reduced capacity to respond to the same toxoid in laterlife. The ability of guinea pigs

to develop tuberculin skin hypersentitivity was suppressed in a large number of animals injected in utero with Old Tuberculin, in a few animals similarly injected with killed tubercle bacilli, and not in any that had been given living bacilli (Weiss, 1958). A partial unresponsiveness in chickens to Shigella paradysenteriae was induced by injecting embryos or newly hatched chicks with appropriate antigen (Friedman & Gaby, 1960). There is only one reported study of the possible role of tolerance in host-parasite relationships and no significant conclusions can be drawn from it. The injections of newborn mice with two different antigenic fractions of Trichinella spiralis, an intestinal nematode, had no apparent influence on the mean worm burdens arising from later infections (Ewert & Olson, 1960).

C. Specific unresponsiveness to soluble protein antigens: That the principle of tolerance originally demonstrated for living cells could be extended to soluble protein antigens was first shown in rabbits. The work of Hanan & Oyama (1954) gave the lead in this respect. These two authors set out to investigate the effect of early immunisation of the rabbit on the gamma globulin content of serum and in so doing discovered that rabbits given a series of injections of BSA in early life failed to produce antibody at that first immunisation and at a second immunisation some 9 weeks later. Shortly afterwards, Dixon & Maurer (1955) published similar findings. They injected young rabbits 6 times weekly for about 100 days after birth with human plasma or BSA and found them to be specifically unresponsive to challenge for at least the duration of the experiment (10-11 months), long after the disappearance of all detectable foreign protein. By way of contrast adult rabbits similarly treated gave evidence of a depressed immune response only so long as the injected protein was detectable (3-4 months)

Dixon & Maurer (1955) further established the very important point that the unresponsiveness of neonatally injected animals was not due to neutralisation of antibody. On the contrary passively administered antibody disappeared from the sera of unresponsive rabbits with a half-life comparable to the half-life of homologous gamma globulin. This was strong evidence for a central failure of the immune response. Cinader and Dubert, (1955, 1956) injected newborn rabbits with human albumin within 24 hours of birth and at various intervals during the first 19 days of life, the total doses ranging from 20 - 1110/mg. At intervals the animals were challenged with antigen but consistently failed to produce antibody for the duration of the experiment (100 days).

The offspring of does given twice weekly injections of chicken serum for the 4 weeks before term were able to form antibody when challenged at 5 weeks, but littermates which received supplementary injections of antigen during the first 4 weeks of life remained unresponsive at 4 months (Downe, 1955). Negative results were obtained by Cohn (1957) who was unable to demonstrate an inhibition of the antibody forming ability of 10-week old chickens that had received injections of protein antigens as 14-day old embryos, but the doses which he employed were very small and did not exceed 1 mg of protein per embryo. That unresponsiveness could be achieved in chickens was shown by Hasek (1956), who found suppressed precipitin formation in hens that had been exposed to turkey blood as embryos. In an attempt to assess the amount of antigen required to induce a state of specific unresponsiveness in rabbits, Smith & Bridges (1956) injected crystalline BSA into new-born animals in graded doses ranging from 1 - 200 mg protein.

Reinjected at 4 - 8 months none of the rabbits given a neonatal dose of 20 mg or more responded with antibody production. There were variable responses from those given less than 20 mg and some indication of a 'partial tolerance'.

These studies were essentially qualitative and did little beyond demonstrating that it was possible to achieve an immunologically unresponsive state to soluble proteins by injecting the antigens into very young animals. A more quantitative approach has been made by Smith and Bridges, (1956, 1958, 1959) who injected graded amounts of defined protein antigens into very young rabbits commencing at different times after birth. Their results showed that the duration of unresponsiveness was finite and related to the amount of antigen given at birth, and that it could be indefinitely prolonged by repeated injections of antigen. The same general findings resulted from two independent studies of a similar nature using the chicken as experimental animal. One of these studies was carried out by Wolfe and his colleagues (Wolfe, Tempelis, Mueller & Riebel, 1957; Tempelis, Wolfe & Mueller, 1958, a, b), and the other is described in Section E of this thesis.

Specific unresponsiveness to soluble protein antigens has been demonstrated in rabbits and chickens as described above, and also in mice (Terres & Hughes, 1959; Dresser, 1961) and in guinea pigs (Turk & Humphrey, 1960).

The experimental works cited above have contributed to the evidence which justifies the contemporary view that maintenance of the unresponsive state is dependent upon the continued presence of the antigen and that between the tolerance induced by injecting the embryo or neonate and the suppression of antibody formation brought about by overloading the immunological system with antigen, there is

only a quantitative difference. This topic, and others related to it, are more fully elaborated in the General Discussion of Section E.

10. The 'adaptive period'

Recurrent in the literature pertaining to the experimental reproduction of the tolerant state is the concept of an 'adaptive' or 'induction' period during which tolerance can be induced by means of an appropriate stimulus. The idea originated in Burnet and Fenner's postulate of self-marker components recognisable only during the plastic developmental stage of the immunological system when it was capable of adapting itself to the antigens in its immediate environment. With maturation of the system this ability was lost and any 'foreign' material finding its way into the body evoked a normal response. It was implicit in Burnet and Fenner's proposition that the enzymic changes induced in the malleable phase of the immunological system should be heritable and transmitted to future generations of cells. Tissue homograft studies of actively acquired tolerance lent support to the idea of an adaptive period for it was found that the response of animals to foreign homologous cells changed in the course of development from tolerance to immunity. The changeover was not abrupt but was represented by a gradual transition from a complete tolerance to a normal immune response for a given antigenic stimulus. Waning of the power of the antigen to confer tolerance was followed by a gradual strengthening of its ability to produce an immune response. Between the two phases came a 'null' period when the effect of the antigen was nil, neither tolerance nor immunity resulting.

(Billingham, Brent & Medawar, 1956b).

Naturally there were species differences in respect of the duration of the adaptive period. For the mouse and the chicken the time of birth and hatching marked the end of the tolerance conferring phase, but in the rat tolerance could be successfully induced even two weeks after birth, so that in this species the adaptive period extended well beyond intrauterine life (Woodruff & Simpson, 1955). At the other extreme Schinkel and Ferguson (1953) had found that foetal lambs of 80 - 117 days were fully capable of rejecting homografts at the adult rate, a finding which has recently been confirmed by Moore and Rowson (1961). For rabbits and guinea pigs the adaptive period also appeared to end before term (Eglahl, 1958).

It was partly this evidence of an apparently genuine adaptive period, a time in the developmental stages of the immunological system when a recognition pattern for foreign material could be elaborated, which prompted the use of embryos or neonates in experimental studies with soluble protein antigens. Systems of this type, using single antigens, were particularly suited to quantitative investigations of the variable factors relating to tolerance. The demonstration that the duration of the unresponsive state is finite and related to the size of the inducing dose of antigen, and that unresponsiveness can be prolonged by renewed injections of antigen, has cast doubt upon the validity of thinking in terms of an 'induction period' unique to the immature immunological system, at least so far as soluble protein antigens are concerned. Rather may it be a question of the relative susceptibilities of immature and adult lymphoid cells to the paralysing effects

of excessive quantities of antigen. The problem is one that requires careful experimental analysis, but the designing of appropriate investigations is hampered by the genuine need to distinguish between the doses of antigen needed for the inception and the maintenance of the unresponsive state (Medawar, 1961). Elsewhere in this thesis (Section E, General Discussion) is proposed a method for the quantitative analysis of tolerance to defined protein antigens. It is considered that this method would be a suitable one to use in determining the amounts of antigen required to produce unresponsiveness with increasing age.

II. Tolerance and immunological theory.

It was stated earlier that the idea of an adaptive period in relation to tolerance was an offshoot of Burnet and Fenner's self-marker concept devised to account for the non-antigenicity of body components. The concept was an integral part of the adaptive enzyme theory of antibody formation, originally formulated in 1949 by Burnet & Fenner and further elaborated, but not basically modified, by Burnet in 1956. It was this theory that predicted immunological tolerance as a consequence of prenatal injection of appropriate antigen, and stimulated intense experimentation with far-reaching consequences. The general theory turned out to be in many ways inadequate and incapable of formal proof and was duly discarded, being succeeded by 'selection' theories of antibody formation. The first of these, the natural selection theory of Jerne (1955), was in some ways equivalent to Ehrlich's side chain' hypothesis (cf. Witebsky, 1954) in that it postulated the existence of a multiplicity of natural serum antibodies to meet all antigenic contingencies. In

the later clonal selection theory of Burnet (1959) the antigen played an active part in stimulating the proliferation of the particular clone of cells whose surfaces carried the complementary pattern for the determinant groups of the antigen. This theory took cognisance of the fact that maintenance of the unresponsive state required the continuous presence of appropriate amounts of antigen and made no plea for an adaptive period but argued rather for a difference in the susceptibility of immature and mature antibody forming cells to paralysis by large amounts of antigen (Burnet, 1961). The clonal selection concept of immunological response, though by no means free from criticism, does attempt to integrate tolerance in a general pattern of immunological reactions, and any theory which does not give account for tolerance is clearly inadequate. As Medawar (1961) has pointed out "Tolerance, like the secondary response and the nature of immunological 'memory' has become something of a testing ground for theories of the immune response."

12. Programme of work undertaken

The main experimental work described in this thesis was concerned with attempts to induce specific unresponsiveness to defined protein antigens and to make some quantitative assessment of the variable factors associated with the induction, duration and measurement of the unresponsive state. In most instances, the chicken was the experimental animal used, but one experiment was carried out on pigs. The defined protein antigens used in these studies were two fractions of bovine serum prepared in highly purified form by Armour Laboratories - bovine serum albumin and bovine gamma globulin.

At the time when the experiments with chickens were begun it was known from the work of Hanan & Oyama (1954) Dixon & Maurer (1955), Cinader & Dubert (1955, 1956), Downe (1955) and Smith & Bridges (1956) that it was possible to make rabbits specifically unresponsive to foreign serum proteins by injecting large amounts of the antigen into the neonatal animal, and Hasek's (1956) report had indicated a similar finding for the chicken. There seemed to be no real doubt that specific unresponsiveness to soluble protein antigens and actively acquired tolerance of tissue cells were merely different expressions of the same basic phenomenon of immunological tolerance. Like acquired tolerance, the state of unresponsiveness was induced by exposing the neonatal animal to antigen, exhibited a characteristic specificity and, very important, represented a central failure of the immune response. Although all but one of the demonstrations of unresponsiveness had been made in rabbits, there seemed no reason why it should not be applicable to other species, for actively acquired tolerance had been effectively demonstrated by laboratory methods in mice, rats, chickens and rabbits (Billingham & Brent, 1956) and under natural conditions in cattle (Anderson et al. 1951).

The wide and important biological implications of the new aspect of immunology which immunological tolerance represented made it imperative to learn as much about the basic phenomenon as possible. There was already evidence from work with homografts and from Smith's and Bridges' (1956) study of unresponsiveness in rabbits that tolerance was not an 'all-or-none' phenomenon, but that varying degrees of tolerance could be achieved. Tolerance therefore

seemed susceptible to a quantitative type of investigation, at least from the point of view of determining the amount of antigen required for its induction in different species. Clearly the most satisfactory way to tackle this problem would be to use as simple an immunological system as possible, the type of system where only one antigen was involved, to eliminate the variables inherent in cellular systems with their multiple complex antigenic components. For this purpose a study of specific unresponsiveness with a single purified protein seemed particularly suited, both because of the simplicity of the system and because of the readiness with which quantitative measurements could be applied to it.

There were several reasons for choosing the chicken as an experimental animal in these studies. Little work had been done with simple protein antigens in the domestic fowl, the system was amenable to quantitative investigation and the chicken, relatively immature at hatching, gradually develops a very considerable ability to produce precipitins (Wolfe & Dilks, 1948). It is a matter of relative simplicity to have access to the developing embryo and large numbers of hatching eggs, day-old chicks and young adult birds are usually readily available from commercial poultry dealers. Events proved the choice to have been a good one, for the experimental work on the chicken gave results which confirmed the extreme usefulness of the domestic fowl to any investigation of the quantitative aspects of the induction and maintenance of tolerance. In this species even small amounts of precipitating antibody can be measured with considerable accuracy if suitable analytical techniques are adopted, and the unresponsive state, though specific, is short-lived thereby eliminating

the need for protracted experiments as in the case of the rabbit.

During the course of the work on immunological unresponsiveness it was necessary to digress from the main theme in order to examine certain aspects of serological behaviour of chicken antibody. This was essential in order to establish the validity of the analytical methods being used to measure precipitating antibody in chicken sera. Some experiments were also carried out with chemically altered protein antigens in the fowl (Section D) and a few experiments on the suppression of the immune response by a cytotoxic drug (Section F). The relevance of these experiments to the main theme of this thesis is described in the introductory passages of the respective sections.

SECTION B

MATERIALS and METHODS

1. *Antigens*
2. *Trace iodination of protein antigens with* ^{131}I
3. *Preparation of chemically altered protein antigens*
4. *Preparation of antisera*
5. *'Versene' as a complement-inhibitor*
6. *The precipitin reaction*
7. *The determination of total N*
8. *Radioactive iodine and its measurement*
9. *Intravenous injection of chicken embryos*
10. *Experimental animals*
11. *Abbreviations*

I. ANTIGENS

ANTIGENS

Only protein antigens were used in the experiments described in this thesis.

With very few exceptions, which are duly indicated, two fractions of bovine plasma proteins were used. These were bovine serum albumin (BSA) and bovine gamma globulin (BGG). In most instances the fractions were purified products obtained from the Armour Laboratories, Eastbourne, England. Specifications for these two antigens, as indicated by Armour, are given below.

Bovine Plasma Albumin, crystallised

Globulins ----------< 0.01%
Ash ----------< 1.0%
Moisture ----------< 5.0%

This is a highly purified protein, employed as a reference protein in amino acid assays and in ultracentrifugal and electrophoretic studies.

Bovine Gamma Globulins, Fraction II

Ash ----------< 3.0%
Moisture ----------< 5.0%
Globulins by electrophoresis > 97.0%

Very occasionally the required fractions were prepared in the laboratory from pooled whole bovine serum. Both BGG and BSA were obtained either by $Na_2\ SO_4$ fractionation essentially as described by Kekwick (1938, 1940), or by fractionation of serum with $(NH_4)_2\ SO_4$, (cf. Kabat & Mayer 1948 p. 461).

After dialysis the salted-out serum proteins were centrifuged for one hour at 1500 g and then filtered under pressure. Those to be injected into animals were further

passed through a Seitz filter. Solutions to be used within a short time (up to 7 days) were kept at refrigeration temperature of 5°C, whereas those not immediately needed were held in deep freeze conditions at - 20°C. No preservative was added to solutions in either case.

While no attempt was made to assess the electrophoretic homogeneity of the protein fractions obtained by the salting-out procedure, it was appreciated that these fractions did not attain the high degree of freedom from contamination by other fractions that was the case with the commercial products of Armour Laboratories.

With minor exceptions, which are noted as they occur, no other purified protein antigens were used in these series of experiments. Use was made, however, of quantities of BSA and BGG that had been subjected to chemical modification of the protein molecule. A separate description of such altered proteins is given later (p. 43).

Adjuvanted antigens

Adjuvants are substances used to enhance or stimulate antibody response. Two methods of adjuvanting have been widely used. One involves the precipitation of protein antigens by potassium alum as described by Proom (1943). In the second method the antigen is prepared as a water-in-oil emulsion containing a stabiliser for the emulsion (Freund, 1947). The addition of heat-killed mycobacteria to such emulsions markedly improves their adjuvant effect. If no bacteria are incorporated in the preparation the adjuvant is said to be 'incomplete'.

The mode of action of adjuvants is not completely clear but it is thought that they achieve their effect by a general stimulation of the reticulo-endothelial system,

and by slowing down the rate of release of antigen from the injection site, so providing the antibody-forming centres with more prolonged stimulation.

The above two methods of adjuvanting were employed in experimental work in which the antigenicity of chemically altered proteins was studied in the chicken.

(i) Alum precipitated antigen

The preparation of alum-precipitated antigen was based on the method of Proom (1943) as described by Francis, Mulligan & Wormall, (1954) P. 284. Equal volumes of 2% solution of protein antigen and of 10% potassium alum were mixed, and the pH brought to 6.5 with 2N-NaOH. The precipitated antigen was separated by centrifugation and washed twice with 0.9% NaCl, using a volume twice that of the precipitate. Finally, it was suspended in saline and made to the same volume as the original protein solution. This gave a 2% suspension ready for intramuscular injection.

(ii) Antigen in a water-in-oil emulsion

(Incomplete Freund's adjuvant) To equal volumes of a 4% aqueous solution of antigen and of liquid paraffin was added sufficient 'emocithine' (a commercial emulsifying agent) to give 2g per 100ml of mixture. The mixture was thoroughly homogenised and was judged to be in a stable emulsion when a small volume placed on a water surface remained discrete, and did not disperse. The antigen concentration was taken to be half that of the original antigen solution because of the approximately two-fold volume increase. Preparations were freshly made on the day of injection and were given by the intramuscular route.

Trace-labelled antigens

Because immune precipitation is in many instances a reaction between two proteins not chemically distinguishable, it is impossible to carry out a complete analysis of the precipitate for antibody and antigen unless one of the two components possesses a marker (whether element or group) that is capable of accurate determination. In such circumstances the precipitate can be analysed for total N and for the identifiable component (usually antigen) by virtue of its marker. The amount of the other component in the precipitate is then determined by subtraction.

Marked antigens were first used in 1902 (crab blood containing haemocyanin, and milk containing casein) to demonstrate that antigen took part in the precipitin reaction and was actually present in the precipitate (Heidelberger, 1956). The use of marked antigens was extended to specific analytical procedures by various workers, using iodoalbumin, haemocyanin, haemoglobin, and proteins conjugated with dyes (cf. Boyd, 1956, p. 685-6). The amount of these antigens in specific precipitates was measured by determining the iodine, copper, iron and dye stuff content respectively. It is, of course, possible even with an unmarked antigen, to carry out an analysis in the antibody excess region of the precipitin curve on the assumption that in this zone all added antigen is precipitated. Determination of total precipitate N and subtraction of antigen N added gives values for antibody N and allows antibody/antigen N ratios in the precipitate to be calculated. This was the method that was successfully applied to the study of precipitin systems by Heidelberger & Kendall (cf. Kabat & Mayer 1948). This type of procedure

suffers from the limitation that it is not readily possible to carry the analysis into the antigen excess region, because the assumption of complete precipitation of antigen no longer holds true and it is necessary to have some means of determining how much antigen is left in the supernatant.

It also breaks down completely when the test antigen is similar to but not identical with the immunising antigen, for with such heterologous systems only cross reacting antibody is measured and precipitation of antigen is therefore never complete.

With the advent of suitable methods forlabelling proteins with radioisotopes a new and valuable method was at hand for this type of precipitin analysis and considerable advances in analytical immunochemistry have been made using antigens and antibodies trace-labelled with various radioactive isotopes such as ^{131}I, ^{32}P and ^{35}S (see review by Wormall, 1952). Radioactive iodine has been particularly used in work of this type involving protein antigens because of the relative ease with which proteins may be iodinated and because iodoproteins are not unphysiological. (Knox & Endicott, 1950; McFarlane, 1957). As a result of the iodination procedure the iodine becomes substituted in the tyrosine residues to give 3:5 diiodotyrosine groupings and is not rapidly removed under physiological conditions. Provided that the amount of iodine introduced is not in excess of 1% there is no marked change in the physico-chemical and immunological properties of the protein (Pressman & Sternberger, 1950; Francis, Mulligan & Wormall, 1951a), and at 1% iodination only a negligible proportion of the protein molecules will contain no iodine (Butement, 1948). It has been demonstrated experimentally that

trace-labelling of protein antigens with ^{131}I does not alter the quantitative precipitin curve produced with antisera to the native protein (Eisen & Keston, 1949; Singer & Campbell, 1952).

Talmage & Maurer (1953) have further demonstrated that labelled and unlabelled antigen give identical precipitin curves with an antiserum produced by a labelled antigen. They contended that such evidence justifies the assumption that selective precipitation does not occur in the presence of a mixture of the two antigens. Since trace-labelled antigens contain both labelled and unlabelled molecules selective precipitation would render invalid any analysis of antigen in the supernatants of precipitates on the basis of radioactivity. Using the anti-BSA and anti-BGG systems in rabbits they analysed the supernatants in the region of antigen excess for the presence of antigen by radioactivity and by N determinations on the precipitates formed with a calibrated antiserum. Their results showed no significant or consistent difference in the figures obtained by the two methods, which indicated that significant selective precipitation does not occur in the presence of a mixture of labelled and unlabelled antigen molecules.

Considerable use was made of ^{131}I trace-labelled antigens in the experimental work described in this thesis. Particularly were they used to determine the rate and pattern of removal from the circulation of intravenously injected antigen, and to carry out quantitative analyses on immune precipitates. Such tagged antigens were also of great value in assessing the antibody levels of antisera. The preparation of trace-labelled antigens is described in part 2 of this section.

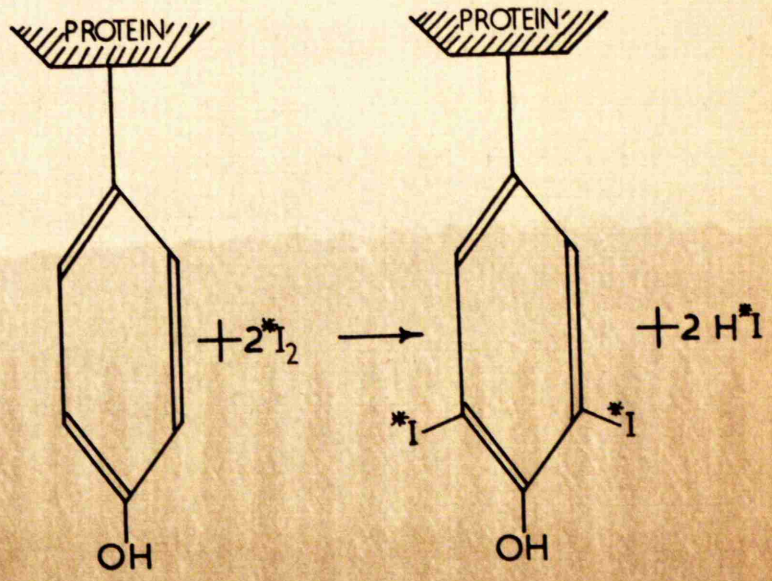
2. TRACE IODINATION OF PROTEINS WITH ^{131}I

Two methods were used for the trace-labelling of protein antigens with ^{131}I. Both were carried out by treating the protein, in slightly alkaline solution, with iodine or iodine monochloride to which had been added a solution of radioactive iodine (as carrier-free iodide). In the preparation of trace-labelled proteins it is desirable that the final concentration of protein should be about 3% since denaturation is more rapid in more dilute protein solutions. Due allowance has to be made for dilution during preparation and during subsequent dialysis for removal of uncombined label.

1. Trace-labelling with free iodine

The method that was used was essentially that of Blum and Strauss (1923) involving addition of a solution of iodine in potassium iodide to an ammoniocal solution of the protein, and was based on the description given by Francis, Mulligan and Wormall (1951) and detailed in Isotopic Tracers (1954) 1st ed. pp. 248-9.

In this method the radioactive iodine is uniformly distributed between the iodine and iodide in the iodinating solution, necessarily entailing a loss of 50% of the radioactive isotope. Further, in the iodination of tyrosine the theoretical maximum utilisation of free iodine is 50%, the other 50% being reduced to hydrogen iodide (see Fig. 1). Thus it follows that the theoretical maximum yield with respect to labelling with ^{131}I is 25%, but in practice one usually gets a net 16%. To improve the yield it would be necessary to convert the iodide to iodine which could then attach to the protein by substitution, but the use of an oxidising system to achieve this could well have deleterious effects on the protein.



IODINATION OF TYROSINE

Fig. 1. *Note that only 50% of the iodine atoms are capable of combining with the tyrosine residues of the protein molecule*

Iodination by the above method is an easy technique, requiring little time, but suffers from the disadvantage of rather low yield with respect to the isotope. The quantities and concentration of iodinating solution generally used were such that BGG was iodinated to about 0.4% and BSA to about 0.5%.

(a) Preparation of iodine solution: A solution of iodine in potassium iodide, of suitable concentration, (N/10), was prepared by saturating N/10 KI with finely powdered elementary iodine. This solution was allowed to stand, with occasional shaking, for 48 hours, after which the excess iodine was filtered off. The solution remaining was approximately N/10 with respect to iodine, and therefore contained 12.7mg free iodine per ml. A more exact figure for iodine concentration could be obtained by titration with N/10 sodium thiosulphate.

From the iodine in potassium iodide solution a volume containing the appropriate amount of free iodine was measured out, and to this was added a solution of ^{131}I (as Na ^{131}I). Following the immediate equilibration between iodine and iodide the solution contained the required amount of radioactive iodine (plus, of course, radioactive iodide which could not react with the protein).

$$I_2 \text{ in } KI/I_2 + I^- \rightleftharpoons I_3^-$$

(b) Preparation of protein solution: The protein was dissolved in 0.15 M - NaCl to give a concentration of 5% and the solution rendered alkaline by the addition of 1 ml of 5N - NH_4OH for every 10 ml of protein solution.

(c) Iodination of the protein solution: The radioactive iodinating solution was added dropwise to the protein solution with constant stirring. A few minutes were allowed for completion of the iodination and the solution

was then brought to pH 7-7.5 by the cautious addition of acetic acid (2N followed by 0.5N, using universal indicator paper as an external indicator). Finally, the labelled protein was carefully transferred to a dialysis sac and dialysed for at least 48 hours at 5°C against several changes of 0.9% NaCl. Following dialysis the labelled protein solution was removed from the sac and centrifuged for 30 - 60 minutes at 1500 g. The radioactivity of labelled proteins was always more than 99% precipitable with trichloracetic acid.

2. Trace-labelling with iodine monochloride

The use of iodine monochloride to trace-label proteins was first described by McFarlane (1958). More efficient labelling and a reduced risk to the operator because of the low volatility of ICl were the advantages claimed for this method. Greater specific activities could be obtained without increasing the amount of isotope used, but the need for ^{131}I completely free from reducing agent called for a radioiodine preparation uncontaminated by thiosulphate. For this particular purpose ^{131}I condensed into N/50 - NaOH can be obtained from the Radiochemical Centre, Amersham.

Thiosulphate-free Na ^{131}I is added to a dilute aqueous solution of ICl which is then converted to hypoiodite, the conversion being carried out by injecting a glycine buffer of pH 8.5 into the ICl solution, and rapidly mixed with the protein solution buffered at pH 9.0. Provided the molar ratio of ICl to protein is greater than 2, 60-80% of the ^{131}I becomes bound to the protein. Even higher efficiencies can be obtained by a separate procedure for oxidation of sulphydryl groups prior to the actual labelling with ICl giving 90% utilisation of the isotope.

Trace-labelling of protein by the ICl method is a reasonably straightforward procedure taking very little time, and, because of the efficiency of labelling, yields a product of high specific activity, even when using comparatively small quantities of isotope. Although in the experimental part of this thesis no controlled comparison of the behaviour of proteins trace-labelled by free iodine and iodine monochloride was carried out, limited experience of proteins labelled by the two methods showed that they did not differ in immunological behaviour.

(a) Preparation of iodine monochloride: The method of preparation followed that given by Vogel (1951) 'Quantitative Inorganic Analysis' 2nd ed. p. 366, Longmans Green & Co.

5.00 g KI (A.R.) and 3.22 g KIO_3 (A.R.) were dissolved in 37.5 ml of water, and to the solution was added 37.5 ml of concentrated HCl and 5.0 ml of CCl_4. The whole was shaken vigorously. The following reaction was thus promoted.

$$2HI + HIO_3 + 3HCl \longrightarrow 3\ ICl + 3H_2O.$$

If the carbon tetrachloride layer did not have a faint pink colour, small amounts of potassium iodide were added until the presence of a little iodine was observed in the carbon tetrachloride. If the colour in the carbon tetrachloride layer was more than a faint pink some potassium iodate solution was added to convert some of the iodine into iodine monochloride. This solution contained approximately 147 mg of iodine per ml as iodine monochloride. (Accurate determination of iodine content could be carried out by addition of excess potassium iodide and titration of free iodine with sodium thiosulphate). Aqueous solutions of

iodine monochloride are stable for months in the presence of a high concentration of chloride ions and a slightly acid reaction. Stock solutions of 4.2 mg and of 0.42 mg of I as ICl were obtained by 1 in 35 and 1 in 350 dilutions of the prepared ICl. The concentrated ICl solution was first partially diluted with molar NaCl, then almost neutralised with N - NaOH, and finally adjusted to N/100 with respect to HCl and molar with respect to NaCl.

From the stock solution of ICl was measured out a volume containing the appropriate amount of iodine as ICl, and to this was added the solution of carrier-free ^{131}I (as Na ^{131}I thiosulphate-free). A volume of 0.1ml of ICl solution containing 0.42mg I/ml as ICl, used to label 20 mg of albumin, will give one atom of I per molecule of protein, assuming 100% incorporation. To achieve a molar ratio of more than 2, three times the equivalent amount of ICl was used for labelling. Even on the basis of 100% uptake of this greater amount of ICl the protein would still not achieve 1% iodination, and would in fact be approximately 0.6% iodinated.

(b) Preparation of buffer solutions: Two glycine buffers were used in conjunction with the labelling process, one (B) for solution of the protein (pH 9 - 9.5) and the other (A) for conversion of ICl to hypoiodite (pH 8.5). The composition of these buffers is given below.

(A) 9 ml M-glycine in M/4 NaCl + 1 ml N - NaOH
(B) 8 ml M-glycine in M/4 NaCl + 2 ml N - NaOH

(c) Preparation of protein solution: The most convenient method for preparing the protein solution was to dissolve the protein in a volume of glycine buffer B sufficient to give a solution of 5% concentration.

(d) Iodination of the protein solution: Following addition of ^{131}I the ICl solution was converted to the hypoiodite, a step that appeared to be essential for substitution of iodine in the benzene ring of tyrosine, and one that is indicated by loss of colour of the ICl. The conversion was carried out by addition of glycine buffer A to the ICl until the colour disappeared whereupon it was immediately mixed with the protein solution. The labelled protein was thereafter completely dialysed and centrifuged as described in the free iodine labelling method. Such labelled preparations never contained any activity not precipitable by trichloracetic acid.

3. PREPARATION OF CHEMICALLY ALTERED PROTEIN ANTIGENS

A number of experiments were carried out to study the response of the chicken to chemically altered antigens. In these experiments bovine serum albumin was altered by one of three techniques - iodination, bromination and the introduction of a phenylureido group - by the methods outlined below.

Controlled iodination and bromination of proteins results principally in the uptake of the element by the tyrosine residues of the protein molecule (Wormall, 1930; Kleczkowski, 1940). Although it is probable that the rest of the molecule is also affected in some way this does not seem to be of significance from the point of view of serological specificity for the reaction between most iodinated proteins and their antisera can be completely inhibited by 3 : 5 - diiodotyrosine (Wormall, 1930).

In the treatment of proteins with phenyl isocyanate to produce phenylureido-proteins, the introduced groups go mainly to the free amino groups of lysine, reducing the serological cross reaction with untreated protein and conferring a new specificity (Hopkins and Wormall, 1933).

1. Iodination

The modified Blum and Strauss method described by Wormall (1930), involving treatment of an alkaline solution of the protein with a solution of iodine in potassium iodide, was applied to the iodination of protein. 150 ml of a solution of BSA in distilled water containing 10 g of protein was treated with 75 ml of 5N - NH_4OH, and to this alkaline protein solution was added the iodinating solution of N/10 I_2 in KI. The addition was made from a burette, a few ml at a time, with shaking after each addition, until free I_2 was detectable by boiled starch five minutes after

the last addition. The total amount of iodine solution so added was 270 ml. Sufficient 5N - acetic acid was added to give maximum precipitation of the iodinated protein which was centrifuged off, washed with distilled water, and redissolved at pH 7.5 with the aid of 2N - NaOH. The iodinated protein was reprecipitated with acetic acid a number of times until no free iodine or iodide was present in the supernatant. This was tested for by treating the supernatant with a few drops of N - KIO_3, dilute HCl and boiled starch solution. It was found necessary to carry out 3 or 4 reprecipitations in order to free the protein from unbound iodine. Finally the iodinated BSA was dissolved in normal saline at pH 7.5 with the aid of 2N - NaOH, and the solution was thoroughly centrifuged to remove all insoluble material. The iodine content of iodinated proteins prepared in this way was of the order of 10%.

2. Bromination

Bromination of serum albumin was carried out using the iodination technique but by substituting saturated bromine water for the I_2 in KI solution. Tests for free bromine were made by adding a little KI solution to the boiled starch preparation. After bromination of the alkaline protein solution had gone to the extent that free bromine was detectable five minutes after the last addition of bromine water, the brominated protein was precipitated by saturation with $(NH_4)_2\ SO_4$. Several reprecipitations were carried out until the supernatant contained no more than a trace of free bromine.

In carrying out the bromination procedure on 3.6 g of BSA contained in 100 ml of distilled water it was found that 55 ml of saturated bromine water was needed to achieve full bromination, as indicated by the detection

of free bromine in the protein solution. $(NH_4)_2\ SO_4$ was used for the precipitation of brominated albumin because it was not found possible to precipitate the protein satisfactorily with acetic acid.

3. Formation of Phenylureido Protein

Treatment of protein with phenyl-isocyanate results in the production of phenylureido protein. The reaction is believed to be mainly at the free amino groups of the protein molecule and to yield a substituted urea as follows

$$R\text{-}CH_2\text{-}NH_2 + OCN\text{-}C_6H_5 \longrightarrow RCH_2 - NHCO.\ NH.C_6H_5$$

(cf. Kabat & Mayer, 1948; Boyd, 1956).

The procedure adopted for carrying out this particular chemical alteration was that of Hopkins and Wormall (1933) as described in Kabat and Mayer (1948) p. 500.

To 50 ml of a solution of BSA containing 3.5g of protein was added 100 ml of a phosphate buffer of pH 8.0 :-

250 ml of 0.2 M - KH_2PO_4
plus 234 ml of 0.2 N - NaOH
per litre.

The buffered protein solution was cooled in ice and stirred continuously as 2 ml of phenyl isocyanate was slowly added. The reaction proceeds at pH 8.0 and regular checks were made to make sure that pH did not fall below this value, using BDH universal indicator paper. Any drop in pH was corrected by the addition of 0.2N - NaOH. After stirring for a further period of 60 minutes the mixture was centrifuged to remove the precipitate of diphenylurea, which was washed twice, the washings being added to the original supernatant. Both the initial supernatant and,

to a lesser extent the subsequent washings, possessed a degree of cloudiness that could not be removed either by prolonged centrifugation nor by filtration. The phenylureido protein was precipitated from the supernatant fluid with 0.5N-acetic acid, separated by centrifugation, and redissolved in distilled water with the aid of a little dilute NaOH, the pH being adjusted to 7.5. As the solution was still a little cloudy it was filtered through fine-pored filter paper, (Whatman No. 50 hard). Finally the solution was dialysed in the cold against 0.9% NaCl for 2 - 3 days in preparation for use in animal immunisation and in carrying out precipitin tests.

4. The preparation of fully iodinated protein trace-labelled with ^{131}I

To determine the behaviour of fully iodinated protein antigen when injected by the intravenous route into chickens a radioactive label was incorporated in the chemically altered protein. The isotope used was ^{131}I and the procedure adopted to prepare the trace-labelled antigen was composed of two steps. First of all the native protein was trace-labelled in the usual fashion by the free iodine method, but instead of adding acetic acid to bring the pH to 7 - 7.5 the protein solution was further ammoniated to bring the total amount of NH_4OH added to half the original volume of the protein solution. Full iodination was carried out by the method previously described, the protein solution finally being brought to pH 7 - 7.5 with acetic acid and dialysed in the cold against 0.9% NaCl for 48 hours.

4. PREPARATION OF ANTISERA

Nearly all the antisera used in analytical work were produced in birds immunised by the intravenous injection of a single dose of antigen of the order of 40-50mg/Kg body weight, an amount adequate for the production of reasonable quantities of precipitating antibody (Brown & Wolfe, 1954). Blood was collected during the height of the antibody response between 7 and 11 days after injection of the antigen. Volumes of blood ranging from 5 - 20 ml were taken at any one time depending on the size of the bird and the number of bleedings made. Blood was obtained by venesection, by cardiac puncture, or by exsanguination, in which latter case about 30 - 50 ml of blood was collected. When the same bird was bled on several consecutive or alternate days, the amount removed daily did not exceed 10ml. Wherever possible, overnight starvation preceded blood sampling. A few antisera were obtained after a secondary response had been elicited by a further intravenous injection of antigen some weeks after the original one. Sampling times and quantities of blood were more or less the same as described above.

Once formed, the clots were separated from the walls of the containing bottle and left overnight at room temperature; then the expressed serum was centrifuged to remove cells and particulate matter. Sera were stored in universal bottles in volumes of up to 20ml each, and kept at refrigeration or deep freeze temperature (4°C or -20°C) until required for use. No form of chemical preservative was added to any normal or immune serum. When ready for testing the antisera were thawed out at room temperature or at 37°C and centrifuged (30 minutes at 1500g) before use. In the case of pools of antisera these were sometimes prepared before, and sometimes after, storage at low temperature.

In certain experiments the immunising antigen was not given intravenously in solution but was presented as an adjuvanted preparation of alum-precipitated-antigen or as antigen in incomplete Freund's adjuvant, and was given by the intramuscular route. The number of injections given was variable but is specified in the appropriate experiments. The antisera so produced were not used in any quantitative analytical work on chicken precipitin reactions but were studied only in relation to the needs of the experiment of which they formed part.

5. 'VERSENE' AS A COMPLEMENT-INHIBITOR

In all sera, both normal and immune, there is present in variable amount a nitrogenous component known as complement that is capable of becoming 'fixed' to specific precipitates formed as a result of the interaction of antibody and antigen. Where precise quantitative analysis of the immune precipitate is carried out, it is of importance that some measure be adopted to prevent complement N from taking part in the precipitin reaction. In chicken precipitin systems, complement N fixation is successfully inhibited by versene, a chelating agent that binds Ca^{++} and Mg^{++}, the cations that play a vital role in the activities of complement (Gengozian & Wolfe, 1957).

Chemically, versene is ethylenediamine-tetra-acetic acid (EDTA), known also as complexone and sequestric acid. The reagent used in all experimental work was the disodium salt (General Purpose Reagent) supplied by Hopkin and Williams, Essex, England. Versene binds Ca^{++} and Mg^{++} more strongly than do anions like citrate and pyrophosphate, but the formation of a chelate between a divalent ion such as Ca^{++} and Mg^{++} and versene leads to the release of H^{+} with a resultant acidification of the reaction mixture unless adequately buffered (Levine, Cowan, Osler & Mayer, 1953). It is necessary to control pH around 7.0 for the sake of the precipitin reaction and also because the chelating efficiency of versene diminishes drastically below pH 7.0. The use of a suitable buffer system is thus indicated to maintain the pH values within the desired limits.

With regard to the total amount of versene required to chelate serum Ca^{++} and Mg^{++} final concentration figures ranging from 0.003M to 0.019M have been reported (Maurer & Talmage, 1953a) but for the chicken system, Gengozian & Wolfe, (1957) found values of 0.005M and 0.0025M to be fully

effective. In the chicken precipitin system the situation is somewhat complicated by the need for a concentration of 8% NaCl in the reaction mixture to ensure complete precipitation of antibody (Goodman, Wolfe & Norton, 1951). The use of a proper salt solution to adjust the NaCl concentration of the whole reaction mixture would have meant an increase in the reaction volume, which leads to undesirable dilution effects on the serum antibody level (Gengozian & Wolfe, 1956). This difficulty was overcome by making the versene solution 16% with respect to NaCl. The versene-NaCl solution was added in equal volume (1.0ml) to the antiserum prior to the introduction of antigen into the reaction mixture, permitting chelation of serum Ca^{++} and Mg^{++} before the immune precipitate was formed, and raising the NaCl concentration of the serum plus versene mixture to 8%, neglecting the small quantity of NaCl in serum. Following thorough mixing of serum and versene, and after 2-3 minutes' incubation at room temperature, 1.0 ml of antigen in 8% NaCl was introduced. The final volume of the reaction mixture was then 3.0 ml. Though this was the pattern that was evolved after some experience with versene, certain differences in the order of addition of reactants and in final volumes are noted in some earlier experiments.

The addition of versene (final concentration 0.01m) to an M/15 phosphate buffer of pH 7.4 caused a fall in pH and it was necessary to add 10 ml of N-NaOH per litre of solution to restore the pH to 7.4. The incorporation of 160g of NaCl per litre of solution brought about a further fall in pH that was again corrected to pH 7.4 by N-NaOH, about 10 ml being adequate.

A careful check on the pH value of the reaction mixture was made immediately on addition of the versene-NaCl to the antiserum, and after addition of antigen. In

both instances the pH was greater than 7.0. Further checks made during incubation and refrigeration indicated that the pH value remained fairly stable. The same stock solution of versene-NaCl was used for quantitative and qualitative precipitin tests in most of the experiments discussed in this thesis. Periodical checks were made on the reaction of the solution, which was found to change very little on storage at -20°C, and if considered necessary, small volumes of N/10-NaOH were used to raise the pH to 7.4. Such volumes were always minimal and of the order of 2-3 ml per litre.

6. THE PRECIPITIN REACTION

THE PRECIPITIN REACTION IN LIQUID MEDIA

An animal immunised with a soluble protein antigen produces antibodies that are known as precipitins because the visible result of their in-vitro action on the homologous antigen is to bring about its precipitation in the form of an insoluble antigen-antibody complex. The amount and composition of the precipitate obtained from a given volume of an antiserum is dependent primarily on the amount of precipitating antibody capable of reacting with the test antigen and secondarily upon the amount of antigen used in the test. In a typical precipitin system the amount of precipitate increases to a maximum as greater amounts of test antigen are added to a constant amount of antiserum, and then undergoes a decrease as shown in Fig. 6 of Section C.

Tests made on the supernatants of precipitates for residual antibody and antigen show that the curve is divisible into three zones. When only small amounts of antigen are added to the antiserum a condition of antibody excess operates. This region is followed by a zone in which antibody and antigen are present in optimal proportions and very little, if any, of either component appears in the supernatant. This is the "equivalence zone" and approximates to the point of maximal precipitation. Finally, the other extreme is reached where the amount of antigen present is in excess of available antibody. This constitutes the zone of antigen excess in which the antibody-antigen complexes tend to be soluble and thus precipitation is less than maximal. Because of this the zone is also referred to as the "antigen inhibition zone."

In determining the precipitin content of an antiserum two types of test are available for general use - the qualitative and quantitative precipitin tests.

1. The qualitative precipitin test

In this test decreasing amounts of antigen, generally in five-fold steps, are added to a constant volume of antiserum. The proportions used are usually five drops of antigen to two drops of antiserum. Suitable controls are included, and for chicken precipitin systems a NaCl concentration of 8% is essential. The results of the test are read after an incubation period of 1 - 2 hours at 37°C with or without additional overnight refrigeration. An assessment is made of the amount of precipitate present and the following conventional scoring system used to describe the results.

sl. tr.	.....	slight trace
tr.	.....	trace
±	.....	a definite precipitate of increasing amount.
+	.....	
++	.....	
+++	.....	
++++	.....	

Because of the essentially arbitrary nature of interpretation of amounts of precipitate, the test is limited in its application to the determination of the presence or absence of a particular precipitating antibody or antigen, but is of some value in comparing relative precipitating abilities of several sera provided they are all tested at the same time and the results recorded by the one person. The qualitative test also provides a useful preliminary to the setting up of a quantitative test because it indicates the extent of the precipitin curve so enabling the quantitative test to be

carried out with the appropriate antigen increments.

Similar in some ways to the qualitative precipitin test is the so-called "interfacial test" in which a standard amount of antigen is added to serial doubling dilutions of antiserum so that an interface is formed between the two components. Essentially the result obtained is a 'titre' i.e. the last dilution of serum which is capable of giving a visible precipitate with antigen (e.g. 1/1280). To some extent this test is semi-quantitative, but it gives no guide to the span of the precipitin curve. In chicken systems non-specific precipitation is prone to occur unless the medium contains at least 1.8% NaCl. No use was made of the interfacial test in any of the experiments described in this thesis.

2. The quantitative precipitin test

It is impossible to obtain information concerning the actual amount and composition of the precipitate throughout the precipitin curve without undertaking quantitative analytical procedures. Early workers were restricted in their investigations because of the difficulties involved in differentiating between antibody N and antigen N in precipitates. As explained previously this difficulty was overcome by the use of antigens possessing a detectable marker in the form of a readily estimable element such as Cu or Fe, or by using proteins conjugated to azo dyes.

However, the foundation of the quantitative precipitin test as a precise analytical method was laid by Heidelberger and Kendall who devised and perfected the technique that has since been so widely adopted (see Kabat and Mayer 1948 Ch. 2 for references and details). Their method of analysis is based upon the assumption of

complete precipitation of antigen in antibody excess. Measured amounts of antigen are added to standard volumes of antiserum until traces of free antigen are detectable in the supernatant by use of a potent antiserum. Determination of total N up to the point of slight antigen excess and subtraction of the total amount of antigen N added gives the antibody N precipitated. With these figures the ratio of antibody N/antigen N may be calculated and where the molecular weights of antibody and antigen are known, the molecular ratio may be determined.

Beyond the point of slight antigen excess reliable quantitative analysis is more difficult to achieve unless it is possible to measure residual antigen accurately, for in this region of excess antigen the assumption of complete precipitation is no longer valid.

Using the method of Heidelberger and Kendall, or one of its modifications, extensive investigations of many precipitin systems have been carried out by various workers, and much of what is currently known about quantitative immunochemical aspects of precipitation has arisen from such work.

The application of isotopic tracer techniques to immunological studies considerably facilitated quantitative immunochemical investigations. The use of trace-labelled antigens and antibodies permits their detection and quantitative determination by radioactivity measurements. Extension of analysis of the precipitate into the region of antigen excess has thus become a matter of relative simplicity and has yielded more precise details about the nature of the immune precipitate in moderate and extreme antigen excess.

Without exception the quantitative precipitin tests described in the experimental section of this thesis made

use of ^{131}I-trace-labelled antigens. Detailed analysis of the precipitates was obtained by carrying out total N determinations and specific activity measurements on either the precipitates or the supernatant solutions, and in some cases on both.

In carrying out a quantitative precipitin test it is advisable to run a preliminary qualitative test to establish the range of the precipitin curve and the region of maximal precipitation. The following description is representative of the type of quantitative precipitin test used for studying chicken anti-protein systems. Apart from the use of versene to inhibit complement N, and the maintenance of a NaCl concentration of 8% in the reaction medium, the test resembles the standard quantitative precipitin test used in mammalian systems.

In all instances the test was performed in duplicate, occasionally in triplicate. All reagents to be used were thoroughly centrifuged, most particularly the antigen and the antiserum, and then into each of several centrifuge tubes was measured 1.0 ml of antiserum (or other measured volume). (At this point it is important to take suitable action to prevent complement N from becoming "fixed" to specific precipitates. In mammalian systems the serum is either heat-inactivated at 56°C for 30 minutes or is treated with an unrelated AgAb precipitate which absorbs the complement N. However, in the chicken system inhibition of complement N is best achieved by chemical means). 1.0 ml of versene containing 16% NaCl was added to the serum, the two were well mixed and allowed to stand for a few minutes before the addition of 1.0 ml of antigen solution in 8% NaCl, after which the tube contents were again well mixed.

Included in the test were suitable controls containing saline instead of one or other of the active components, and where necessary normal serum controls. All tubes were placed in a water bath at 37°C for 1 - 3 hours, after which time they were removed to a refrigerator for overnight storage.

The following day the tubes were centrifuged at 0°C for 30 minutes at a force of 1500 g. Supernatants were then removed by Pasteur pipette and retained while each precipitate was washed with 0.5 ml of ice-cold 8% NaCl and recentrifuged. The washing process was repeated and both washings were added to the original supernatants.

Total N determinations of the precipitates were determined by the Markham modification of micro-Kjeldahl or by a colorimetric technique, and antigen N estimated by radioactivity measurements on the precipitates or supernatants. From the figures so obtained it was possible to determine the composition of the precipitate at all points on the curve and to obtain other very useful information about the amounts of added antigen that were precipitated in the three major zones of the curve. This work is described in detail in the experimental section of this thesis.

THE PRECIPITIN REACTION IN AGAR GELS

The gel diffusion techniques of Oudin and Ouchterlony (Oudin 1952) for studying precipitin systems have been widely applied in recent years, particularly in investigations concerned with homogeneity of antigens and cross reactions between related immunological systems. In these techniques the precipitin reaction takes place in an agar gel through which one or both reactants are allowed to diffuse. The precipitate, once formed, is more or less fixed, and when more than one antigen-antibody system is involved, more than a single boundary of precipitate may develop, because of different diffusion rates of the antigens and of specific characteristics of the individual antigen-antibody system.

There are two basic methods of gel diffusion that are commonly used.

1. The tube test (Oudin)

In this test only one component of the precipitin reaction diffuses through the gel, and the name "single-diffusion" test is therefore sometimes applied. Antiserum is mixed with warm molten agar which is then allowed to solidify in small tubes. Antigen solution is layered over the gel and as the antigen diffuses into the gel an opaque boundary of precipitate is formed. A "double-diffusion" tube test has been described by Pope, Stevens, Caspary and Fenton (1951) and by Feinberg (1957) in which antibody and antigen are separated by a layer of neutral agar into which they can both diffuse.

2. The plate-test (Ouchterlony)

This method depends upon the principle of double-diffusion. A thin layer of molten neutral agar is poured into a petri-dish and allowed to solidify. Small wells

are bored at some distance apart and into these are placed the antigen and antibody solutions so that they can diffuse towards each other. Where they meet in suitable concentrations a line of precipitate is formed. If the antigen contains more than one active fraction and if corresponding antibodies are present in the antiserum, then several lines, each corresponding to a particular antigen - antibody system, will be formed.

The type and amount of information that can be obtained from antigen-antibody diffusion in agar plates is dependent upon the particular arrangements of wells employed, and many variable patterns have been devised. (Wilson & Pringle, 1954; Korngold, 1956; Allison & Humphrey, 1959; Feinberg, 1957, 1960).

The plate diffusion test was used to study some of the precipitin systems described in this thesis. These plates were prepared from 1.0% agar dissolved in 0.9% NaCl according to the following formula:

10 g agar (New Zealand)
1000 ml 0.9% NaCl
10 ml 1:1000 merthiolate

The saline was brought almost to boiling point, the agar added, and the preparation then kept at gentle boiling temperature until all the agar had fully dissolved as shown by the disappearance of the agar globules. Before cooling 10 ml of merthiolate was run in. This amount was found to be adequate for preventing contamination of the plates by micro-organisms or moulds. The molten agar was poured into universal bottles in 15 ml lots, the agar permitted to cool, and the bottles capped. These bottles of agar were stored at room temperature.

Preparation of Plates: When required for use the agar in the universal bottles was melted by standing the uncapped bottles in boiling water for a few minutes. The contents of each bottle were then poured into a petri-dish lying on a completely level surface. Once the plated agar solidified the petri-dish lids were replaced and the plates put in the refrigerator for at least 2 - 3 hours. By doing this the wells in the agar could be more cleanly cut.

For reproducibility of results in agar diffusion it is essential that distances between wells should be constant. To achieve this, pattern templates were prepared from small blocks of wood or paraffin wax into which small pins were fixed at accurately measured distances. By pressing the template on to the surface of the agar the pattern was imprinted, the mark left by each pin head representing the centre of a well. Using a cork borer passed through a close-fitting, rigidly clamped, vertical sleeve, small cylindrical plugs of agar were removed by suction to form the wells. The bottom of each well was sealed with a drop or two of molten agar to prevent the well contents from seeping away between the agar layer and the base of the petri-dish.

The wells were then ready to receive their contents, a volume of 2 - 3 drops being sufficient to fill each well. The petri-dish lids were placed over the plates which were then set in an incubator at 27°C. at a high humidity to await development of the precipitate lines. The choice of temperature at 27°C was not specifically designed for the agar plates but was that at which the laboratory incubator was constantly run. High humidity, achieved by keeping an open tray of water in the incubator, was necessary to prevent excessive drying-out of the plates. Lines of

precipitate usually appeared in 18 - 24 hours and certainly by 48 hours in most chicken systems. In cases where it was considered necessary to recharge the wells equal amounts of antiserum and antigen were used, for the use of disproportionate amounts of either reactant causes distortion of precipitate lines and results in poor definition (Wilson and Pringle 1954). Results were generally read at 24 hours, noted on specially prepared record sheets, and checked again at 48 hours when any interim developments of significance were added to the records. Precipitate lines remained more or less fixed for a few days after which some haziness and loss of definition was noticeable.

The template and well pattern most commonly used was based on the conventional 6 - membered ring with a seventh well at the centre, as shown in Figure 2.

Although chicken precipitin tests conducted in liquid media require a NaCl concentration of 8% for complete precipitation of antigen-antibody complexes in comparative plate tests using agar prepared in 1, 4 and 8% NaCl the best results were obtained with the 1% NaCl system, the precipitate lines in higher NaCl concentrations being less concise and more poorly defined.

Slight modifications of technique were introduced from time to time to meet particular circumstances. Clarification of agar by the method of Feinberg (1956) using Hyflo supercel was found to be a somewhat tedious and time consuming procedure, and equally good clarification was obtained by filtration of molten agar under pressure, which yielded a product clear enough for most practical purposes. The incorporation of various dyes into the agar did not improve resolution of lines and the procedure was not pursued. Loss of water from the wells by evaporation

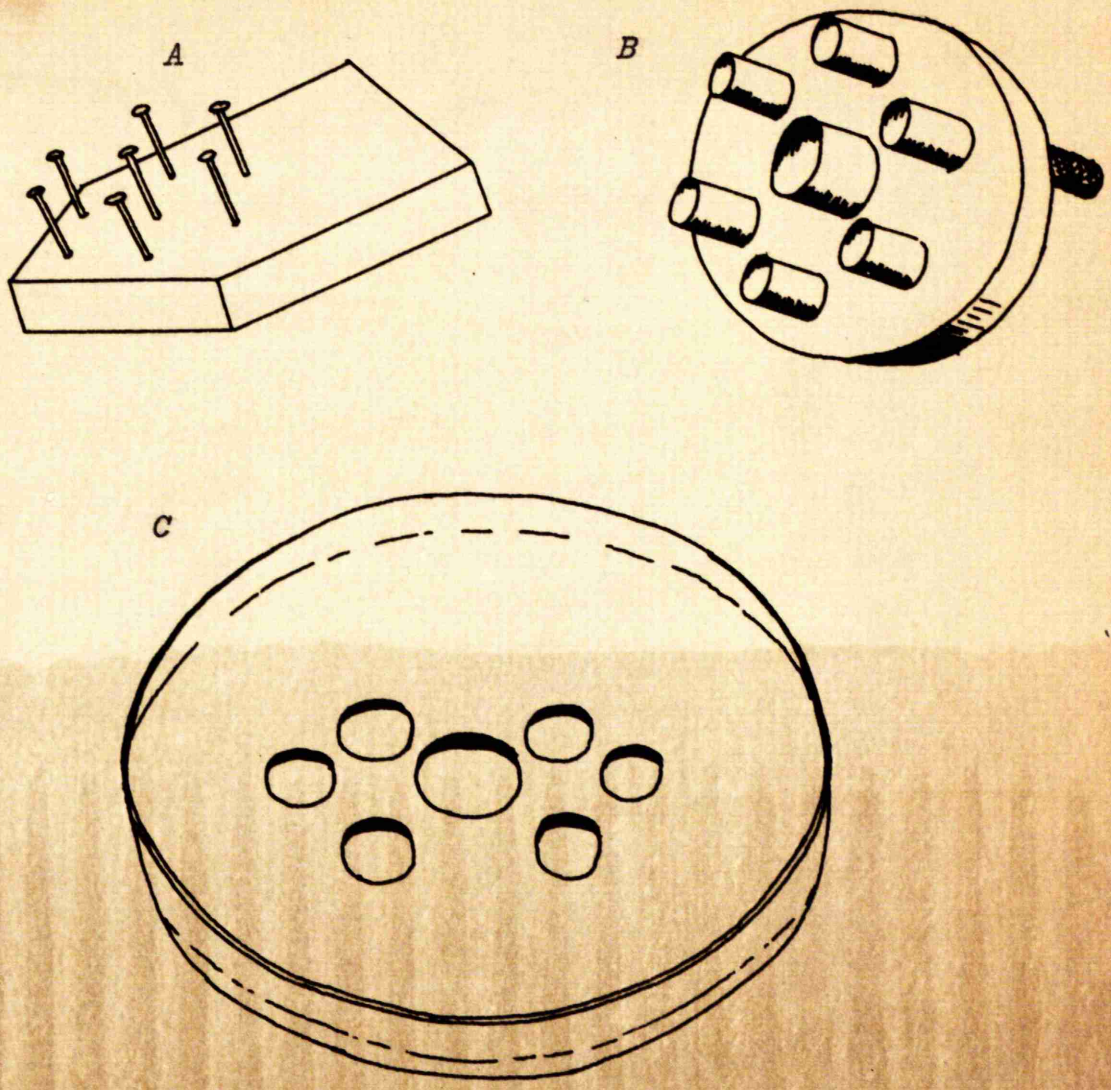


Fig. 2. A. *Template for well pattern*

B. *Gel cutting tool devised by Feinberg*

C. *Wells cut in agar in Petri-dish*

during incubation was avoided by covering the open petri-dish with a circle of 'parafilm' wide enough to overlap the dish, so that when the lid was placed on there was virtually an airtight seal.

In general the sensitivity of agar gel diffusion techniques for studying precipitation is several times less than that of the fluid tube test. In liquid media the antigen and antibody when present in correct proportions can combine quantitatively whereas in solid agar only fractions of the reactants are available for the formation of precipitates. But given standard conditions with a repreducible well pattern and applying due caution in the interpretation of results, the plate agar gel diffusion test has considerable value in qualitative comparison of precipitin systems and is particularly useful in studying the relationship and cross reactions of different immunological systems.

7. THE DETERMINATION OF TOTAL N

Two methods were used for determining total N. These were the Markham modification of the micro-Kjeldahl technique (Kabat & Mayer 1948) and a colorimetric procedure based on the biuret reaction.

1. Markham modification of micro-Kjeldahl

This method is particularly designed for the analysis of samples containing 10 - 100 µg N. The principle is the same as that of the micro-Kjeldahl method. The protein, in the sample to be analysed, is decomposed by digestion with conc. H_2SO_4 and a catalyst, the N being converted to $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ solutions are then transferred to a distillation apparatus, treated with an excess of NaOH, and the NH_3 steam-distilled into saturated boric acid which is finally titrated with N/70 HCl.

The following are details of the analytical technique as applied in the experimental work relating to this thesis.

The volume of conc H_2SO_4 used in each digestion was 1.0 ml. The acid itself was of M.A.R. grade.

The catalyst employed was the conventional micro-Kjeldahl catalyst, consisting of

8 parts K_2SO_4
1 part $CuSO_4 \cdot 5H_2O$
1 part SeO_2

A knife-point quantity (c.0.5g) of catalyst was used for each digestion.

As an indicator the mixture described by Kabat & Mayer (1948) was used i.e.

Stock solution A: 1% methylene blue in water

Stock solution B: saturated solution of twice-recrystallised methyl red in 95% ethanol.

Stock solution C: 15 ml. of stock solution A mixed with 125 ml of stock solution B.

For carrying out titrations 2.0 ml of solution C was mixed with 200 ml of boric acid solution, a fresh mixture being prepared each day.

3.0 ml volumes of the boric acid indicator mixture were used for collection of the distilled NH_3, each distillation taking only a few minutes. Titration of the ammonium borate by N/70 HCl was carried out using an "Agla" micrometer syringe (Burroughs - Wellcome & Co.), keeping the tip of the syringe needle immersed in the distillate. Stirring was effected by means of a stream of nitrogen bubbles, the nitrogen having been previously washed by passage through boric acid solution.

When samples contained more than 100 μg N, 5.0 ml of boric acid-indicator mixture was used, the N/70 HCl was run in from a micro burette, and a small mechanically driven glass propellor used to stir the distillate.

Always before use the distillation apparatus was thoroughly steamed out and blanks were run on all the reagents to make certain they contained no source of N, or alkaline contents. Total blank values of 7 μg N or less were considered satisfactory provided they remained constant. A number of runs were made with standard NH_4Cl solutions to make sure that the apparatus was functioning properly, that a good end-point was achieved with the particular batch of indicator, and that reproducible results were being obtained.

2. Colorimetric estimation of N.

The biuret reaction was the basis of the colorimetric

method used for the determination of protein N. The biuret reagent used was that of Dittebrandt (1948), containing, per litre:

9.0 g NaK tartrate (Rochelle salt)
3.0 g $CuSO_4 \cdot 5H_2O$
5.0 g KI
0.2 N-NaOH (carbonate-free)

The NaK tartrate is dissolved in approximately 400 ml of 0.2N - NaOH. The $CuSO_4$ is then added and dissolved, and is followed by the KI. Finally the solution is made to 1 litre with the 0.2N - NaOH.

The different instruments were used at various times for measuring the optical density of the biuret solutions. They were

a) Hilger Uvispek Spectrophotometer (Wavelength 555 mμ)
b) Unicam spectrophotometer SP 600 (Wavelength 540 mμ)

Each instrument was previously calibrated using protein solutions whose N content had been determined by micro-Kjeldahl analysis, thus providing an optical density - protein N graph for each instrument. Reproducible results were obtained over long periods as determined by recalibration.

Before addition of biuret reagent precipitates were dissolved in 0.2 ml of N/10 - NaOH. The amount of biuret subsequently added varied with the instrument being used but was standard for any one instrument.

The biuret colorimetric method for N determination was particularly useful when analysing large numbers of precipitates, because of the simplicity of the technique, which was none-the-less accurate and sensitive enough to determine as little as 25 μg quite satisfactorily.

8. RADIOACTIVE IODINE AND ITS MEASUREMENT

Radioactive iodine (^{131}I) as a trace-label has been widely used in immunological investigations and has proved of great value in this respect. It is particularly useful because of the ease with which proteins can be iodinated, and it possesses a half-life that is sufficiently long to permit experimental work to proceed without undue concern about loss of activity. Being an emitter of medium energy β particles and of moderately hard γ rays its radioactivity is readily measurable by conventional instruments based on gas ionisation or scintillation in solid crystals. The physical details for ^{131}I are

Half-life (days)	Radiation energy (MeV)	
8.04	0.61	0.36

Supplies of ^{131}I were obtained from the Radiochemical Centre, Amersham, in the form of carrier-free iodide ($Na\ ^{131}I$). When the ICl method was used for trace-labelling the radioactive iodine was obtained thiosulphate-free. The amount of isotope obtained at any one time varied from 5 - 20 mc, the activity being specified for the actual day of labelling, though not all the ^{131}I in any particular batch was used to trace-label antigens.

In handling radioactive solutions and in disposal of waste the standard recommended rules were adhered to. Very active preparations were always handled with remote control manipulators and kept inside "caves" of steel and lead bricks. In trace-labelling procedures full precautions were taken including the use of safety trays, long pipettes, maximum possible shielding and rubber gloves. On completion of the manipulations the regional bench top and adjacent areas were fully monitored. Contaminated glassware and instruments were steeped for a few days in water containing some NaOH to

facilitate removal of traces of labelled protein. Liquid waste, such as the initial dialysate of labelled solutions, was stored in isolation until the activity had decayed sufficiently to permit of drain disposal.

Trace-labelled antigens were used for two purposes - to facilitate the analysis of specific precipitates and for following the removal from the circulating blood stream of intravenously injected antigen. In both cases, but more particularly in the case of quantitative precipitin tests, it was necessary to make certain that all the ^{131}I was in fact bound to protein. This was checked after dialysis and centrifugation of labelled protein by precipitation of an aliquot with trichloracetic acid. With the standard procedures of dialysis for at least 48 hours and centrifugation at 1500 g for 30 - 60 minutes it was always found that the activity of such protein solutions could be wholly precipitated with the protein.

The evidence for the normal physiological and serological behaviour of trace-labelled proteins is discussed elsewhere in this section in connection with the actual trace-labelling procedures (p. 36).

When antigen elimination was being studied it was important to make sure that the experimental animals had access to adequate amounts of inactive iodine to ensure saturation of the thyroid and to limit uptake of ^{131}I. To achieve this purpose a source of iodine was provided for the birds by iodinating the drinking water to the extent of 0.05% with Na or K iodide. Such treated water was presented to the birds for at least seven days before trace-labelled antigen was injected.

The amounts of ^{131}I used for labelling antigens were such that, even with the relatively efficient ICl method, no individual bird received more than 50 μc at any one time. In fact in the majority of instances a much smaller amount of activity was injected. Such small doses were unlikely to have had any serious adverse effect on the general health of the experimental animals, and certainly in normal control birds antibody synthesis was not apparently interfered with.

Measurement of radioactivity

Two different types of instrument were used for the detection and measurement of radioactivity. These were a Geiger Muller counter of the end window variety and a scintillation counter.

The Geiger-Muller counter was of the thin end-window variety, model EW3H supplied by 20th Century Electronics, having a mica foil window of 1.5 - 2.5 mg/cm^2 and of 25 mm diameter. During use the counter was housed in a Panax universal lead castle and connected to a Panax Scaler (Type 100 C).

Serum or plasma samples for counting were pipetted on to small nickel-plated planchets of 1.5 cm diameter (G.E.C.), previously made grease-free by ether/alcohol washing. To promote even spreading of the plasma on the planchet 0.1 ml of "Cetavlon concentrate" (cetrimide B.P. 20% W/V, I.C.I.), was pipetted on to each planchet. Thorough mixing of the sample on the planchet and removal of bubbles, was achieved by directing a jet of air from the nozzle of a fine pipette on to the surface of the sample. Thereafter the sample was evaporated to dryness by the application of gentle heat from a hot-plate. As it is of particular importance that evaporated samples for counting should be of uniform thickness to ensure even distribution

of radioactivity on the planchet surface, all cracked or uneven samples were discarded. Using an end-window counter the greatest volume of plasma that can readily be evaported for accurate counting is 0.25 ml. Thus its ability to detect and measure small amounts of activity is limited by the actual volume of sample that can be counted.

All samples and standards were prepared in duplicate and each counted at least twice. To correct for self-absorption an appropriate aliquot of normal serum or plasma was spread on planchets carrying the standard solution.

The scintillation counter was used much more frequently than the G - M tubes, because being a very efficient dectector of γ rays, it is particularly suited to the measurements of ^{131}I, and samples are counted in liquid form in 5 ml volume. The particular model used was an EKCO type N 550A, containing a thallium-activated crystal of NaI as the solid phosphor. In the earlier work this crystal was of the annular ring type, N 597 model, of cylindrical shape, over which was placed a plastic cuvette containing the 10 ml sample. This was later replaced by a well-type type of crystal (N553A) into which was placed a thin, even-walled glass tube containing the 5 ml sample to be counted.

As the well-type crystal has an efficiency about four times that of the annular ring the sensitivity of the counter was correspondingly increased, and the use of tubes instead of cuvettes facilitated handling of samples during counting.

There was an independent power supply to the photomultiplier and the scaling unit was a Panax 100 C model.

For scintillation counting a net count of at least 2000 was required for each sample, and every sample was counted at least twice. Counts of less than twice the background were considered to be beyond the lower limit for accurate determination.

9. *INTRAVENOUS INJECTION OF CHICKEN EMBRYOS*

In connection with the studies on immunological tolerance to protein antigens in the chicken it was necessary to make intravenous injections of antigen in 14-day old embryos. So far as was possible a uniform technique was adopted and adhered to, but with progressive experience came improvements and modifications, and these are included in the method as described here.

The preparation of antigen for injection is discussed in the protocols of particular experiments, but in general the antigen was dissolved in sterile normal saline, centrifuged, and finally passed through a bacteriological filter. Prior to injection it was raised to a temperature of approximately 37°C.

All instruments, syringes and needles were sterilised in boiling water, and operators and assistants washed and scrubbed their hands thoroughly, rinsing them in disinfectant. In order to see the recipient blood vessel clearly it was necessary for the operator carrying out the injection to have his face within a few inches of the window in the shell. To reduce the possibility of droplet infection reaching the embryo, a surgical face mask was worn by the injector, and by the person drilling the shells.

All operations took place in the room where the incubator was housed. Sources of heat were provided by lamps and electric fires to prevent excessive cooling of the eggs while out of the incubator. A few eggs were removed at a time and immediately candled (transilluminated to determine the presence or absence of an embryo), and all non-fertile eggs rejected. At the large end of the egg, adjacent to the air-space, a pencil mark was made over one of the larger chorio-allantoic vessels. This area of the

shell was swabbed clean with alcohol or tincture of iodine, and a small piece of shell lying over the marked vessel was removed by cutting through the shell with a rotary drill bearing a smooth-edged carborundum stone in the head. The isolated piece of shell was removed with a pair of fine forceps. Care was taken during drilling and removal of the shell not to pierce the underlying shell membrance. The window thus created in the shell measured approximately 1.5 cm x 0.5 cm.

A few drops of warm sterile normal saline placed on the shell membrance effectively cleared it and revealed the underlying chorio-allantoic vessel in clear relief. For the purpose of injection the egg was held at approximately 60^{o} to the horizontal, with the large end uppermost and the window facing the operator. The injection of warm antigen solution was made using a tuberculin syringe and a 5/8" 28 gauge hypodermic needle. The tip of the needle was introduced into the vein at its lower end and slid forward some distance before actual injection, then left in position for a few seconds to allow the injected material full chance to disperse. On withdrawal of the needle there occurred a slight leakback of blood via the point of penetration but this was seldom extensive. When faulty injections were made or if there was a significant amount of blood leakage from the punctured vessel the egg was discarded. During actual injection there was generally some reaction from the embryo in the form of a sudden slight movement.

The injection having been successfully completed the window was sealed. If the removed piece of shell was intact it was replaced and fixed in position using molten paraffin wax. Where the piece of shell was broken a cover was made for the window using 'parafilm' (sheet paraffin wax) the edges being sealed with a heated spatula.

Cellotape was also tried for this purpose but was found to be unsatisfactory as the chicks had difficulty in breaking through the shell at that point at the time of hatching.

Once sealed the egg was replaced within the incubator, with the trephined area uppermost. Although for a single egg the whole procedure lasted little more than a few minutes the necessity of dealing with a few at a time meant that each egg was out of the incubator for about 10 minutes, and, as each of the two compartments held 150 eggs the doors were being opened and closed at frequent intervals over a period of about two hours. As will be explained in the experimental section of the thesis intravenous injection of embryos resulted in a high mortality of embryos and a low hatching percentage. The rather lengthy handling and considerable physical interference associated with drilling almost certainly played a significant contributory role in this respect.

During the 6 - 7 days following injection the eggs were turned at least once daily and to avoid damage to the windows this was done by hand. In retrospect and in the light of more recent experience it seems that this would have been better left undone for once the window comes to lie on the undersurface of the egg there is a tendency for the embryo to fall on to it and to adhere to this area. Such comparative immobility of the embryo would seriously reduce its chances of hatching.

With a little experience the technique of intravenous injection of embryos becomes relatively straightforward and is one that could lend itself to many immunological and other investigations.

10. EXPERIMENTAL ANIMALS

The animal most frequently used in these studies was the domestic fowl; some experiments were also carried out on rabbits and on pigs.

Birds

All hatching eggs, day-old chicks and almost all the mature birds employed were of the Golden Legbar X Light Susex variety, a sex-linked cross whose males are white and females a golden brown. The supplier was the Poultry School of the West of Scotland Agricultural College, Ayr. Day-old chicks were in the laboratory within 2 - 3 hours of despatch from the hatchery, thus neonatal injections could be made within 24 - 36 hours of actual hatching.

Normal poultry husbandry practice was followed, chicks being reared under electric brooders with a progressive reduction in temperature over the first few weeks, and thereafter in an indoor deep-litter house. In later experiments it was possible to house birds in tiered fattening cages, each containing 4 - 10 birds depending on age and size. This method was particularly convenient for separating different experimental groups.

Occasional outbreaks of coccidiosis among young birds were treated by the administration of 33.3% sulphamezathine I.C.I. (Sulphadimidine B. Vet. C.) in the drinking water to give a final concentration of 0.1%. Sporadic bouts of cannabalism occurring amongst birds being kept in rather crowded conditions were controlled by debeaking and by the use of hen spectacles. Apart from occasional isolated deaths young adults and mature birds maintained reasonably good health and productivity.

Other breeds of domestic fowl used for experimental work were Light Sussex, White Wyandotte and Rhode Island Red.

Young adults of the former two breeds were used in studies relating to serological responses to chemically-altered antigens, while culled Rhode Island Red hens from a commercial laying flock were used as a source of normal chicken serum, and very occasionally for the production of immune serum.

Rabbits

These were all adult stock rabbits of the Dutch variety, kept in individual cages under standard animal house conditions.

Pigs

Use was made of the litters of two adult Large White sows to study the immunological effect of neonatal injection of protein antigen. These animals were part of the stock of a commercial piggery and after weaning were reared indoors in a fattening house.

11. ABBREVIATIONS

The following abbreviations are used from time to time in this thesis.

Ab	Antibody
Ag	antigen
As	antiserum
NCS	normal chicken serum
BSA	bovine serum albumin
BGG	bovine gamma globulin
HGG	human gamma globulin
V.S.	Versene in 16% NaCl.

In addition there occur certain physical constants and chemical symbols which conform to accepted convention.

Tables and Figures in Sections C to F are numbered according to the sequence in which they occur, a new sequence commencing at the beginning of each Section.

SECTION C

A STUDY OF THE ANTI-BOVINE GAMMA GLOBULIN (BGG) AND ANTI-BOVINE SERUM ALBUMIN (BSA) PRECIPITIN SYSTEMS IN THE CHICKEN

1. INTRODUCTION

Consequent upon diverging pathways of evolutionary development and specialisation, birds and mammals differ from one another in many respects. Such differentiation is not limited to particular anatomical adaptations, but is apparent also in a variety of physiological and biochemical processes, including some aspects of immunity. It is not surprising, therefore, that certain of the standard laboratory procedures used to measure the immune responses of mammals such as the rabbit, cannot be applied successfully to similar studies in birds without some modification of technique.

Rice (1947, 1948) and Karrer, Meyer and Eddie (1950), have drawn attention to the need for modification of the standard complement fixation test for the satisfactory demonstration of avian antibodies to Salmonella pullorum and to ornithosis virus. Jankovic and Isakovic (1960) have observed that whereas the sera of chickens immunised with rabbit and duck erythrocytes possess good agglutinating titres to these foreign erythrocytes they consistently fail to bring about haemolysis even in the presence of adequate amounts of complement derived from guinea pig and other mammalian and avian sera. This failure they attribute to a peculiar inability of chicken antibody to fix complement and have suggested that a study of immune haemolysis involving avian antibody might well reveal important information concerning the mechanism of complement fixation especially as there occur in chicken and other avian sera natural haemolysins capable of exhibiting haemolysis.

The pitfalls inherent in the use of serological techniques devised for mammalian systems in analysis of avian antisera have been particularly noted by workers interested in precipitin production in the domestic fowl. As a

considerable part of this thesis is concerned with chicken precipitating systems, it is appropriate that the principal findings concerning chicken anti-protein sera be mentioned here.

Hektoen in 1918 recorded his experience with the chicken in immunological studies. He found it to be a prompt and generous producer of precipitins and of greater reliability in this respect than the rabbit. A tendency on the part of chicken antiserum to give non-specific reactions following rapid thawing from the frozen state was observed but this was not apparent when thawing was done gradually. Non-specific reactions were also observed when "interfacial titres" were determined in 0.9% NaCl, but could be avoided by the use of 1.8% NaCl in this test. Wolfe and various workers (1942 et seq.) have made an extensive study of precipitin production in the chicken. Their researches proved this species to be an extremely efficient producer of precipitating antibodies in response to serum protein antigens, potent antiserum being obtained within a few days of a single antigenic stimulation. In the typical response of adult birds to a single intravenous injection of BSA (40 mg/Kg), detectable antibody made its first appearance on the 5th or 6th day after injection of antigen. Peak antibody levels were attained at 8 or 9 days following injection, and were of the order of 250-300 µg Ab N per ml of serum, but peak levels of 500 µg Ab N per ml were not uncommon (Brown & Wolfe, 1954). Once the peak level had been attained, serum antibody concentration declined over a period of 10 days and by the 21st day after antigenic stimulation, circulating antibody had reached very low levels (see Fig. 1). If following the decline and disappearance of serum antibody, a secondary response was induced by giving a further injection of BSA at 40mg/Kg, peak antibody levels were considerably augmented and could rise above 2000 µg AbN/ml of serum (Banovitz & Wolfe, 1954).

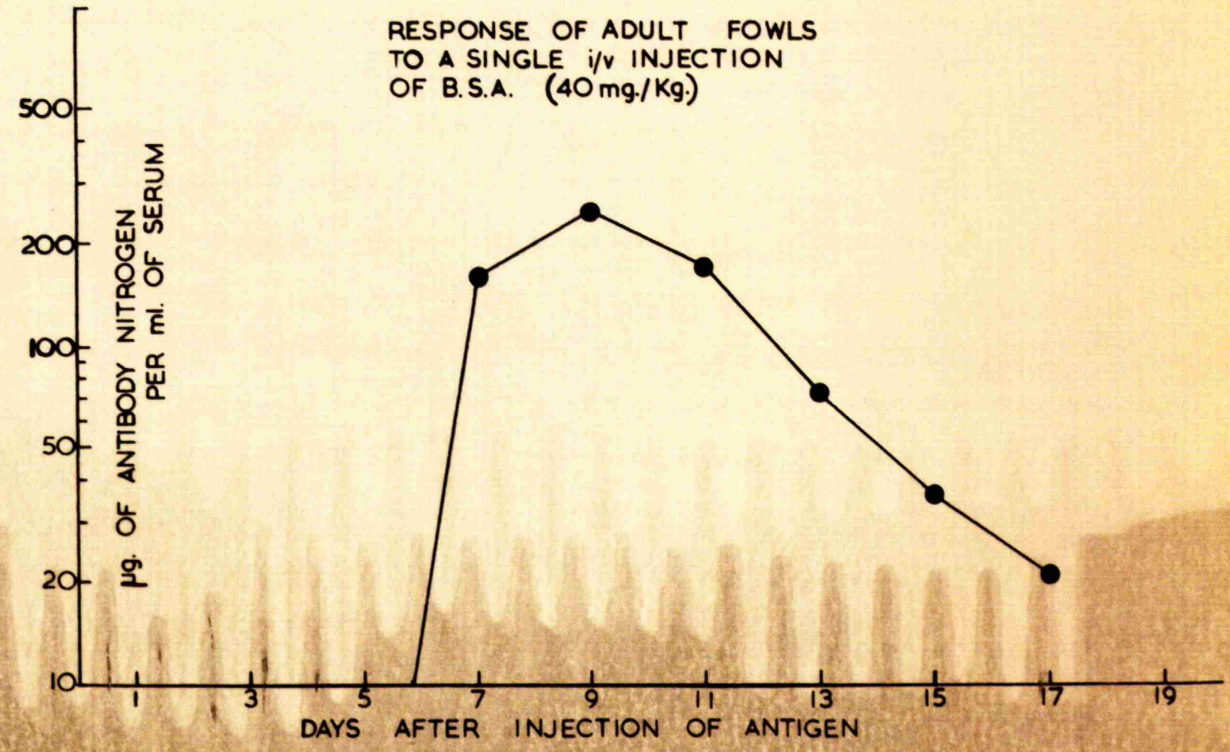


Fig. 1. *Serum antibody levels in adult birds given a single intravenous injection of BSA, 40mg/Kg, on day 0 (data taken from Brown & Wolfe, 1954)*

It was shown by Brown and Wolfe (1954) that no significant increment in peak antibody level, nor in duration of antibody response was achieved by dividing the single dose into three injections given on alternate days. At a standard antigen dose of 40 mg BSA per Kg body weight, the subcutaneous route of injection was as effective as the intravenous, though when 200 mg per Kg was employed, the subcutaneous route was more efficient.

The intraperitoneal route of antigen injection was used on occasion in relation to some of the experimental work described in this thesis and precipitating antibody was regularly produced. From the limited quantitative data available for adult birds, the response appeared to be of the same order as that following intravenous injection.

In their investigations, Wolfe and his colleagues paid particular attention to the physico-chemical conditions affecting the formation of immune precipitates. One of their earliest observations was the rise in interfacial titre which occurred when sera were aged at $4^{\circ}C$, the rise commencing within 24 hours of bleeding and increasing progressively to reach a peak level some 8 - 12 days later (Wolfe, 1942; Wolfe and Dilks, 1946). When quantitative precipitin tests were carried out it was found that the process of ageing produced either an increase or a decrease in the amount of N which could be precipitated, depending on whether the quantitative tests were performed in low or high saline concentration respectively (Gengozian & Wolfe, 1957). During the initial storage period at low temperature, or following freezing and thawing, a gelatinous flocculate appeared in the serum. Apart from the fact that it is apparently not complement, nothing conclusive is known about the nature and significance of this material. Similar findings were reported by

Moody, Cochrane and Drugg (1949) who observed that the turbidity of fresh chicken antiserum increased when measured on successive days.

Directing their attention to the problem of salt concentration and efficiency of precipitation, Wolfe and his associates confirmed the earlier observations of Hektoen concerning the need for 1.8% NaCl when measuring interfacial titres, and from quantitative precipitation experiments, discovered that increasing amounts of N were precipitated as the NaCl concentration of the test medium was increased. After eliminating the possibility of the precipitation of non-specific N at higher salt concentrations, they concluded that at least 8% NaCl is necessary for the measurement of the total amount of antibody in a chicken precipitating antiserum (Goodman, Wolfe & Norton, 1951).

In a chicken anti-HGG precipitin system which they studied, Deutsch, Nichol, and Cohn (1949) reported that up to 50% of their immune precipitate consisted of α globulin, hence they considered fractionation of the antiserum to be essential for the accurate determination of precipitating antibody (Cohn, 1952). However as these workers adopted an immunisation procedure that involved multiple antigen injections, and performed their qualitative and quantitative tests in 0.9% NaCl, it is not possible to compare their results directly with those of Wolfe and his colleagues.

In carrying out quantitative precipitin analyses, it is necessary to bear in mind that complement N can become fixed to the immune aggregate and contribute significantly to total precipitate N. Thus in determining the composition of specific precipitates it is not possible to calculate accurately the amount of antibody N by subtraction of antigen

N from total precipitate N without making some allowance for the presence of complement N. The most satisfactory way to circumvent this complication is to adopt a procedure that will prevent the uptake of complement N by the specific precipitate. Heating mammalian antiserum at 56°C for 30 minutes will successfully destroy the haemolytic activity of complement N and also prevent a considerable portion of its nitrogenous components from coprecipitating with a specific antigen-antibody complex (Maurer & Talmage, 1953a ; Heidelberger, 1956). When this method was applied to chicken antiserum, non-specific precipitation during the test resulted, (Gengozian & Wolfe 1957), and some other means of decomplementing chicken antiserum had to be sought. According to Gengozian and Wolfe, the most satisfactory method is the use of versene (ethylene-diamine-tetra-acetic acid) as a chelating agent to bind the Ca^{++} and Mg^{++} ions essential for complement activity. This complement-inhibiting agent was routinely used in serological analyses described in this thesis. Increasing the volume of a reaction mixture containing undiluted serum (i.e. diluting all the constituents) has no significant effect on the amount of precipitate obtained from a rabbit precipitin system (Kabat & Mayer 1948, p.35). This is far from being the case for chicken precipitin systems where a fourfold increase in the reaction volume can bring about a 25% reduction in the amount of total N precipitated (Gengozian & Wolfe 1956). This effect is considered to be due to the greater dissociability of chicken antigen-antibody complexes.

The phenomena associated with ageing of chicken antiserum -increased interfacial titres, variation in precipitable N, and spontaneous flocculation-have not been recorded in mammalian antisera. Furthermore high NaCl concentration,

far from augmenting the amount of N in the precipitate from, say, rabbit antiserum, brings about a reduction in specific precipitate N (Kabat and Mayer, 1948,p.37). From the experience of various workers, and particularly from the experimental evidence provided by Wolfe and his colleagues, it has become clear that chicken antiserum exhibits particular characteristics which demand special precautions in applying routine serological tests for precipitating antibody.

The ready availability and domesticity of the chicken largely determined the choice of this species for studies in avian serology, but the comparative ability of eight avian species to form precipitins to serum protein has been studied by Wolfe and Dilks (1949). Only the partridge, owl and pheasant were capable of forming precipitating antibody in amounts comparable to the chicken. Both owl and pheasant sera yielded greater precipitates with increasing salinity of the test medium (Goodman & Wolfe,1952) which indicated that in this respect atleast, the behaviour of chicken antiserum is not unique. However, this phenomenon cannot be regarded as characteristic of avian species in general.

Before commencing a series of immunological studies on chicken precipitin systems, using as antigens bovine serum albumin (BSA) and bovine gamma globulin (BGG), it seemed advisable to investigate the nature and characteristics of the precipitin curves obtained with these antigens. This work provided information which proved of considerable value in the formulation of a method of antibody measurement in anti-protein sera.

As much of the available data concerning chicken anti-protein systems had been acquired by qualitative and/or semi-quantitative methods there was felt to be a need for

more precise information, especially concerning the composition of the immune precipitate. In their studies of precipitin production in the chicken, Wolfe and allied workers had modelled their quantitative precipitin test (Gengozian & Wolfe,1956) upon the pattern devised by Heidelberger and Kendall (Kabat & Mayer 1948, p.22). This test involved measurement of the amount of N present in precipitates obtained up to the region of slight antigen excess, the latter being identified as the point at which residual antigen was first detectable by a potent antiserum in the supernatants of the specific precipitates.

While this method yielded reproducible results it lacked the sensitivity required for a critical analysis of the composition of the antigen-antibody complex at various parts of the curve, and could not readily be extended into the antigen excess zone.

Quantitative precipitin tests designed to elicit this type of information were therefore undertaken in both the anti-BGG and anti-BSA systems. In these tests the attachment of a radioactive trace-label to the antigen facilitated analyses of the precipitates and permitted the accurate measurement of very small quantities of antigen. It was moreover possible to continue the analysis into the region of antigen excess.

The same sensitive analytical procedure was applied when, in the case of the anti-BSA system, the pattern of precipitation in various concentrations of NaCl was investigated.

Inhibition of co-precipitation of complement N with the specific precipitate by the disodium salt of versene was studied in the anti-BGG system.

The experiments performed on these various aspects of chicken antiserum protein systems are described in this section.

2. THE FIXATION OF COMPLEMENT NITROGEN BY SPECIFIC PRECIPITATES AND ITS PREVENTION

In mammalian species complement can affect the precipitin reaction by incorporating in the antigen-antibody precipitate as much as 30 to 60 μgN per ml of serum (Heidelberger & Mayer 1942; Cushing and Campbell 1957 p.290). This effect is also noticed in aged sera that have lost their haemolytic activity (Maurer and Weigle 1953). Although complement N is fixed in all three zones of the precipitin curve it is on antigen excess that the most significant effect has been reported (Maurer & Talmage 1953a, 1953b). Elimination of complement N as a co-precipitating factor is thus an essential prerequisite for quantitative analyses of precipitates for antigen and antibody.

By heating mammalian serum at 56°C for 30 minutes, the heat-labile components of complement are inactivated. Haemolytic activity is destroyed and the fixation of complement N by immune aggregates is largely prevented (Maurer & Talmage 1953a, 1953b, Heidelberger 1956). A similar decomplementing effect can be achieved by treating the serum with an unrelated specific precipitate (Maurer & Talmage 1953a, 1953b). When Gengozian and Wolfe (1957) attempted heat decomplementation of chicken antisera non-specific precipitation occurred when the quantitative test was performed in 8% NaCl. However, a satisfactory decomplementing agent was found in versene, ethylene-diamine-tetra-acetic acid (EDTA). This compound chelates Ca^{++} and Mg^{++}, two cations which are essential for complement haemolytic activity, and exerts a potent inhibitory effect on the uptake of complement N by specific precipitates in mammalian systems (Maurer & Weigle 1953, Levine, Cowan, Osler & Mayer 1953).

Applied to the chicken anti-BSA system, versene was reported by Gengozian and Wolfe (1957) to be effective as a decomplementing agent as concentrations comparable to those

used for mammalian sera. A decrease in the amount of total N precipitated from fresh and aged serum was observed when quantitative precipitin tests were carried out in a medium containing versene; the decrease occurred throughout the entire curve, and was apparently greatest in the region of considerable antigen excess.

As a preliminary to its use throughout the quantitative precipitin studies it was felt desirable to check the use of versene as a complement inhibitor. If satisfactory the reagent should bring about a reduction in the total N precipitated but this must be shown to be due not to any interference with specific antigen-antibody precipitation, i.e. the amount of antigen precipitated should be the same whether the complement inhibitor is present or not. This type of experiment is most easily carried out using a labelled antigen so the influence of versene on the chicken anti-BGG system was studied in this fashion.

Antiserum

Antiserum was prepared by immunising each of ten 6-week old birds with 31.0mg BGG * in 0.15 M-NaCl, given as a single intravenous injection. Eight days later the birds were bled and a qualitative test on the sera indicated that all ten birds had responded well and produced good precipitin titres. From each serum was taken an aliquot of 3-4 ml to establish an antiserum pool for the quantitative precipitin test. The pool was held at -20°C until required.

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* *In fact the BGG was trace-labelled with ^{131}I so that its elimination from the circulation could be followed. As explained in Section B (p. 35) the introduction of ^{131}I in trace amounts does not alter the immunological specificity of the protein.*

Versene

When divalent ions such as Ca^{++} and Mg^{++} chelate with the disodium salt of EDTA there is a release of hydrogen ions with consequent acidification of the medium; below pH 7.0 the chelating effect of EDTA is drastically diminished (Levine et al.,1953). It is thus necessary to ensure during the precipitin reaction and throughout the period of incubation that the pH of the medium remains above 7.0. From the stand-point of the precipitin test itself it is important to maintain a near neutral pH. The procedure adopted was to prepare versene as a 0.01 M solution in a phosphate buffer of pH 7.4. Since the quantitative precipitin system in the fowl must be carried out in 8% NaCl, a source of high saline concentration was required, so sufficient solid NaCl was added to the versene solution to give a final NaCl concentration of 16%. The pH of this versene-NaCl solution was found to be a little below 7.0, but was adjusted to 7.4 by addition of a very small volume of 2N-NaOH. Several times during the process of incubation of the precipitin reaction checks were made on hydrogen ion concentration which was found to remain suitably constant.

In the particular case to be described the versene-saline solution was used in the test at a volume equal to the total of all other reactants so that the final effective concentration of NaCl in the precipitin tests was 8%*, and of versene was 0.005M; this concentration of versene lies within the range found to be satisfactory for inhibition of complement. In later tests the procedure was modified by preparing the antigen in 8% NaCl, so permitting direct addition to the antiserum of an equal volume of the versene

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* *This figure of 8% neglects the NaCl content of serum and antigen solutions.*

TABLE 1.

Scheme of Quantitative Precipitin Test with and without versene.

Chicken Anti-BGG System

All volumes in ml

Component	Tube No. 1 abc	2 abc	3 abc	4 abc	5 abc	6 abc
Serum	1.0	1.0	1.0	1.0	1.0	1.0
0.9% NaCl	2.0	1.9	1.8	1.6	1.2	0.4
* Versene (series a & b) OR 16% NaCl (series c)	3.0	3.0	3.0	3.0	3.0	3.0
+ Antigen (ml)	0.05	0.1	0.2	0.4	0.8	1.6
Total Volume	6.05	6.0	6.0	6.0	6.0	6.0

Control Tubes

Component	Tube No. 7 ab	7 c	8 ab	8 c	9 a
Immune serum	-	-	-	-	1.0
Normal serum	1.0	1.0	-	-	-
* Versene	3.0	-	3.0	-	3.0
16% NaCl	-	3.0	-	3.0	-
0.9% NaCl	0.4	0.4	1.4	1.4	2.0
+ Antigen	1.6	1.6	1.6	1.6	-
Total volume	6.0	6.0	6.0	6.0	6.0

* Versene = Versene in 16% NaCl at pH 7.4

Final effective concentration of versene = 0.005 M
of NaCl = 8%

\+ A stock solution containing 800μgN/ml

solution before introduction of antigen.

The Quantitative Test

For the test, tubes were set up in triplicate and designated a,b,c; versene was used in a duplicated series (a and b) while the third set of tubes (series c) contained no versene and acted as controls. The extent of the precipitin curve having been indicated by a preliminary qualitative test, there was prepared a stock antigen solution from which aliquots were removed and added to 1.0 ml samples of serum (see Table 1).

In this way, a condition of antibody excess was established in the tube containing the least amount of antigen, and at the other end of the scale, sufficient antigen was used to ensure a condition of antigen excess. The actual amounts of antigen employed in the test are indicated in Table 1. The antigen was trace-labelled with ^{131}I, and was in fact the same antigen that had been used to immunise the birds.

The scheme of the test is given in Table 1, together with a scheme of all control tubes employed in the test. All reactants having been added, the contents of the tubes were thoroughly mixed and then underwent a 90 minute period of incubation at 37°C. Following overnight refrigeration all tubes were centrifuged at 0°C for 30 minutes at 1500 g; supernatants were then carefully removed by Pasteur pipette and retained for further investigation.

Treatment of precipitates: All precipitates were washed three times with ice-cold 8% NaCl, 0.5ml per washing with a centrifugion period of 30 minutes at 1500 g between each washing. Thereafter, 3.0 ml of modified biuret reagent (Dittebrant, 1948) was added to each tube and the contents dissolved over a period of 30 minutes. Optical densities of these solutions and of several dilutions of stock antigen were determined in the Uvispek

spectrophotometer at a wavelength of 555 mμ. Conversion of optical density values to nitrogen concentration was achieved by reference to a standard calibration curve for the instrument.

A scintillation counter with an annular ring type of crystal was used to measure the radio activity of aliquots of the precipitate solutions, and of various dilutions of stock antigen. Using the specific activity value of the antigen it was possible to determine the amount of antigen N in the specific precipitate, and subtraction of the antigen N value from total precipitate N gave a figure for antibody N, (antibody N + complement N in the tubes containing no versene).

Treatment of Supernatants: Following the initial centrifugation, the supernatants were removed and submitted to a qualitative test for the presence of residual antibody by addition of a standard antigen solution, and for residual antigen by addition of a known positive antiserum. In these qualitative tests the pattern adopted was of the usual type and is summarised below.

A. Versene System

4 drops of supernatant + (i) 1 drop V.S.* + 1 drop AS = Test for Ag

(ii) 1 drop V.S. + 1 drop Ag = Test for Ab

(iii) 1 drop V.S. + 1 drop 0.9% NaCl = Control

* (V.S. = versene + NaCl solution)

B. Non-Versene System

As above, but 16% NaCl substituted for V.S.
Tests were read after 90 minutes at 37°C.

TABLE 2.

Total N and Ag N precipitated with and without versene

All values in μg N

Tube No	Total N		Ag N	
	Versene a & b	Non Versene c	Versene a & b	Non Versene c
1	208	254	37.0	35.7
	210		37.1	
2	273	328	62.6	65.6
	303		65.8	
3	328	380	92.1	92.0
	340		91.9	
4	332	400	119	116
	345		116	
5	(410)	365	147	128
	320		147	
6	260	310	166	144
	(360)		177	

Results of Analyses

Results of the analyses of the precipitate for total N and for antigen N are shown in Table 2 and in Figs. 2 & 3. All the control tubes gave essentially negative results indicating that non-specific precipitation either of antibody N or antigen N did not occur under the conditions of the test. Agreement between total N analyses on duplicates (series a and b) was completely satisfactory with the exception of two figures in tubes 5 and 6 which were very much out of line (values in parentheses in Table 2), and which were not used in plotting Figures 2 and 3. The other total N values for tubes 5 and 6 fell more naturally into the curve and correlated well with a later analysis on the same serum pool. Duplicate values (a and b) for antigen N were in good agreement throughout the curve and the figures obtained for the untreated series c coincided with them at all points except 5 and 6 where somewhat lower values were obtained in the untreated system.

Discussion

In the region of antibody excess and throughout the zone of maximum precipitation the results in Figs. 2 and 3 completely vindicate the use of versene as a complement inhibitor i.e. the presence of versene brings about a reduction in the total N precipitated without altering the amount of antigen N precipitated. The position with regard to points 5 and 6 (antigen excess) is less satisfactory, for the total N figures of the untreated series approximated to those obtained for the versene treated series (Fig. 2). It is noticeable, however, from Fig. 3, that a simultaneous fall in the amount of antigen N precipitated occurred in the untreated system which suggests that there may have been a loss of specific precipitate during the thrice-repeated washing. To some

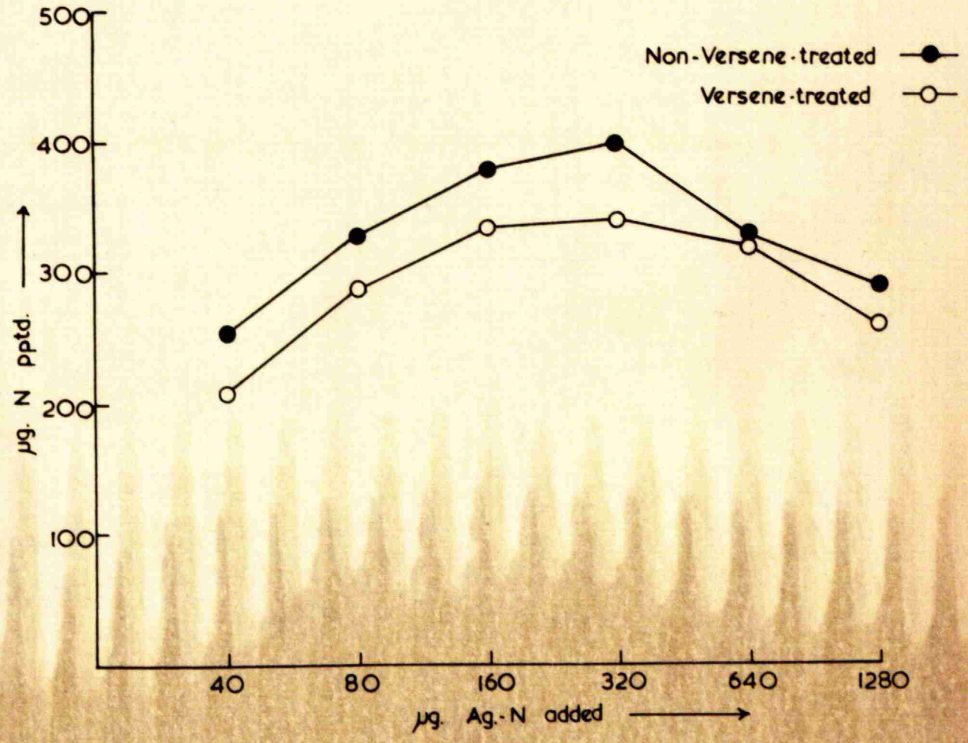

Fig. 2. *Total N values for precipitates obtained from a chicken anti-BGG system with and without the use of versene as an inhibitor of complement*

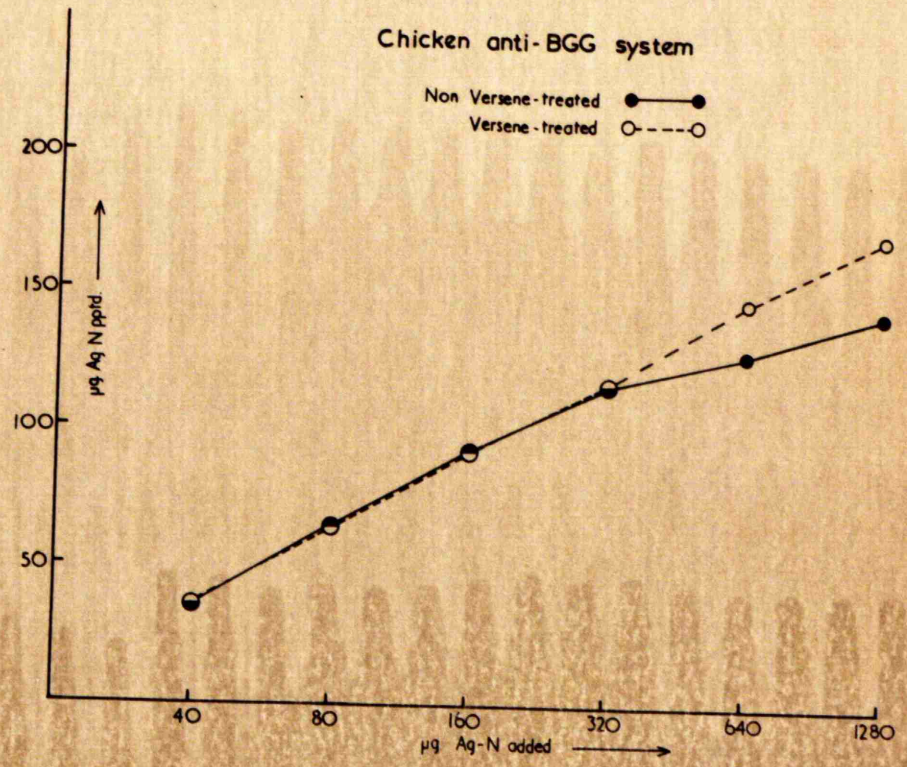

Fig. 3. *This figure shows the amount of antigen N precipitated in the versene-treated and untreated systems. The smaller amounts precipitated in the untreated system may have been due to loss of some specific precipitate during washing*

TABLE 3.

Results of qualitative precipitin tests on supernatants

Test for	System	Tube No. 1	2	3	4	5	6	Control
Ag	Versene	-	+	+	+	++	++	-
			-					
	Non-versene	-	+	+	+	++	++	-
			-					
Ab	Versene	+	-	-	-	-	-	-
	Non-versene	+	-	-	-	-	-	-

extent this possibility is confirmed by the qualitative tests on the supernatants which indicated that both systems had equivalent amounts of residual antigen at points 5 and 6. (Table 3)

In an investigation of versene-treated and non-versene-treated chicken anti-BSA systems, Gengozian and Wolfe (1957) found that reduction in the amount of precipitated N was most apparent in the region of extreme antigen excess, which was similar to the results obtained in studies on complement inhibition in rabbit precipitating systems. While such was apparently not the case with the chicken anti-BGG system, if it can be assumed that some precipitate was lost at points 5 and 6, then obviously the true results would have given more close agreement with those of Gengozian and Wolfe. Notwithstanding the variation in results in the region of antigen excess, it was clearly shown by this experiment that the effect of versene, employed in the chicken anti-BGG system under the conditions described, was to inhibit the coprecipitation of a non-specific nitrogenous substance, most probably complement

The experiments of Gengozian and Wolfe and the results of this experiment using BGG, emphasise the necessity for making sure that complement nitrogen does not contribute to the precipitate obtained when antiserum and antigen react together. Versene proved a reliable and satisfactory agent in this respect and did not produce any anomalous results provide that the pH of the reaction mixture was greater than 7.0 (Levine et al, 1953). In view of these finding subsequent chicken precipitin reactions were performed in the presence of versene in a reaction mixture of pH 7.4, the final effective concentration of versene in the reaction mixture being 0.005M.

3. QUANTITATIVE ANALYSES OF THE ANTI-BGG AND ANTI-BSA PRECIPITIN SYSTEMS

I. THE ANTI-BGG SYSTEM

Antiserum

The immune serum was drawn from a pool essentially similar in content to that used to study the efficiency of versene as an inhibitor of complement. It was prepared in 6-week old birds by the single intravenous injection of 31.0mg of BGG and bleeding 8 days later.

Antigen

The trace-labelled immunising antigen was also used for performing the quantitative test, accurate dilutions of a stock solution providing a range of amounts of the antigen for testing against standard volumes of antiserum. Total N analysis of antigen solutions by the Kjeldahl method (vide infra) permitted correlation of N content with radioactivity determined in the EKCO annular ring scintillation counter.

Sample	*Vol (ml) analysed*	*µgN*	*Results expressed as N content of 1.0 ml stock Ag*	*Mean (µg N)*
Stock Ag	*0.25*	*600*	*2400*	
1/8 " "	*1.0*	*296*	*2368*	*2380*
1/100 " "	*1.0*	*23.68*	*2368*	

The Quantitative Test

The test followed the basic patterns already outlined in the description of studies on complement nitrogen, and the scheme of the tests is given below.

Scheme of Quantitative Test

Component	*Tube Number*								*Serum control*	*Ag control*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*		
Serum	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0* +
Versene *	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*	*2.0*
0.9% Nacl	*0.5*	-	*0.75*	*0.5*	-	*0.75*	*0.5*	-	*1.0*	-
Antigen	*0.5*	*1.0*	*0.25*	*0.5*	*1.0*	*0.25*	*0.5*	*1.0*	-	*1.0*
Final Vol.	*4.0 ml in each case*									

* *The versene was prepared in 16% NaCl1 the final effective concentration of versene in the test being 0.005M and of NaCl being 8%*

\+ *NCS*

The test was run in quadruplicate, the four sets of tubes being designated a, b, c, d.

After addition of all reactants the tubes were all well shaken to mix the contents, which underwent the standard incubation of 90 minutes at 37°C, and then overnight refrigeration at 5°C. Centrifugation (1500 g for 30 mins. at 0°C) was followed by removal of supernatants and a thrice-repeated washing of the precipitates with 0.5ml volumes of ice-cold 8% NaCl. The washings were added to the original supernatants.

TABLE 5.

Data for quantitative precipitin test on chicken anti-BGG system

(All N values in μg)

Tube No	Ag N added	Total N pptd	Ag N pptd	Ab N pptd	% Ag pptd	Ab N / Ag N
1	18.6	140	18.0	122	96.8	6.8
2	37.2	229	35.9	193.1	96.5	5.4
3	74.4	324	69.5	245.5	93.4	3.7
4	148.8	364	96	268	64.5	2.8
5	297.5	357	118.7	238.3	39.9	2.0
6	595	362	131	231	22.0	1.8
7	1190	295	166	129	13.9	0.8
8	2380	199	108	91	4.5	0.8

The values for Total N and Ag N represent means of quadruplicate samples. Nagative results were obtained for serum control and antigen control tubes.

Analysis of Precipitates

10ml volumes of 2N-NaOH were used to dissolve the precipitates, and Kjeldahl nitrogen analyses were performed on 4.0ml aliquots. Results obtained for total precipitate N are given in Table 5: Antigen N in the specific precipitates was determined by radioactivity measurement. Antibody N was, the difference between total precipitate N and antigen N in the precipitate. From these three sets of figures certain other information was derived, viz., the percentage of antigen precipitated at each point on the curve, and the N ratio of Ab/Ag throughout the curve. All the relevant figures are given in Table 5 and plotted graphically in Fig. 4.

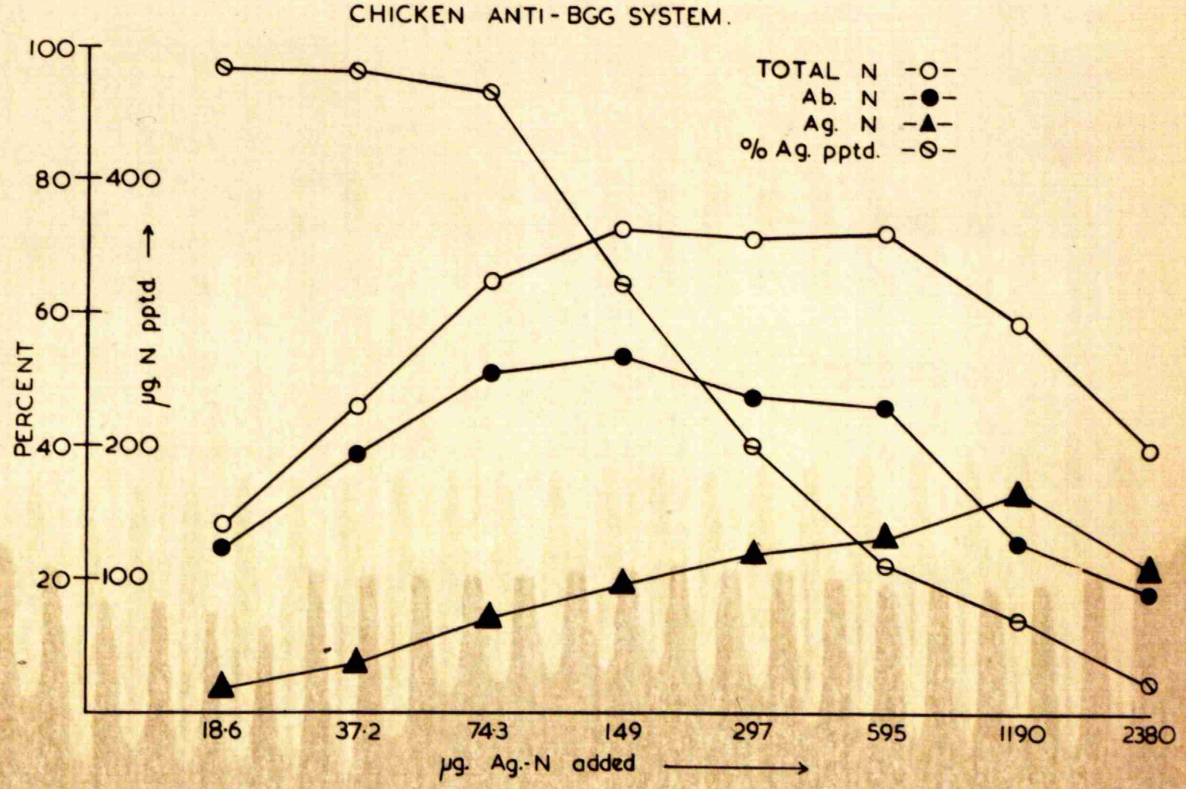

Fig. 4. The chicken anti-BGG precipitin curve

TABLE 6.

Data for quantitative precipitin test on chicken anti-BSA system

(N values in μg)

Ag N added	Total N pptd	Ag N pptd	Ab N pptd	%Ag N pptd	Ab N / Ag N
3.8	74	3.7	71	95.5	19.1
7.6	138	7.6	131	99.7	17.2
15.3	194	14.7	179	96.0	12.2
38.2	298	26.2	272	68.6	10.4
76.4	302	26.7	275	35.0	10.3
153	240	27.6	212	18.1	7.7
478	182	23.2	159	6.1	6.9
624	185	20.6	164	3.3	8.0

Triplicate samples were used to determine mean figures for total N and antigen N. Negative results were obtained in antigen control tubes while the semn control value did not exceed 12 μg N

2. THE ANTI-BSA SYSTEM

The results presented here are part of the experiment concerning the effect of electrolyte concentration on specific precipitates (see p 114). All the data given below refer to the test performed in 8% NaCl. The quantitative test procedures were the same as for the BGG system, but the range of amounts of antigen employed was extended at the lower end of the scale. Table 6 contains all the relevant analytical results and the corresponding curves are given in Fig. 5.

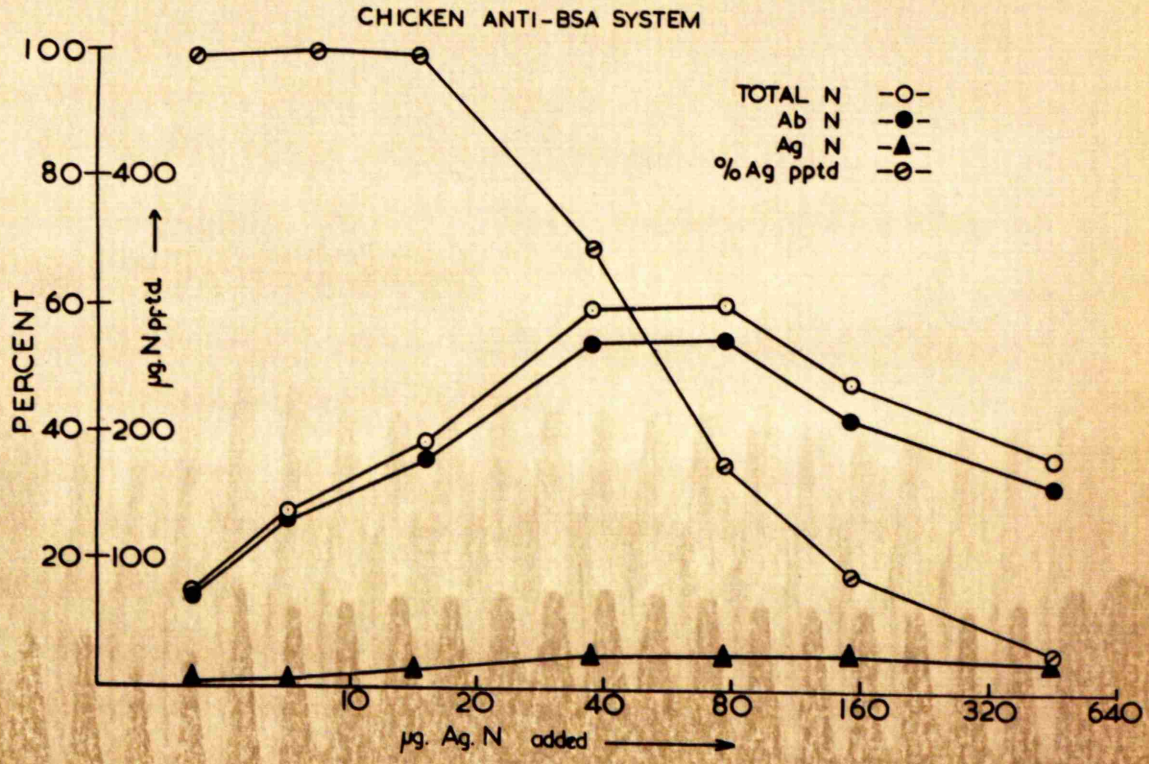

Fig. 5. The chicken anti-BSA precipitin curve

DISCUSSION

Shape of the precipitin curve The precipitin curve records the amount of precipitate obtained from standard volumes of antiserum when these are permitted to react with increasing amounts of antigen. Typically in the case of mammalian antisera, the amount of precipitate varies according to the relative quantities of antigen and antibody which are present in the system as shown in Fig. 6. In such a characteristic curve can be discerned three distinct zones:

a) antibody excess zone in which virtually all the available antigen is precipitated,

b) equivalence zone where both antigen and antibody are almost totally precipitated,

c) antigen excess zone in which the antigen-antibody complex tends to be soluble and the total precipitate is less than the maximal.

However, with mammalian precipitating antisera, the actual shape of the curve varies with different immunological systems and three principal types are recognised (Cushing & Campbell, 1957, p 288) These are depicted diagrammitically in Fig. 7.

1. The rabbit anti-ovalbumin type of precipitin curve is characterised by a steep rise to a sharp peak in the region of maximal precipitation and a fairly rapid decline in antigen excess.

2. The horse anti-toxin type, the most striking feature of which is inhibition of precipitation in the antibody excess zone. In this curve too, the peak is rather narrow and is followed in antigen excess by a sudden drop in the amount of precipitate obtained.

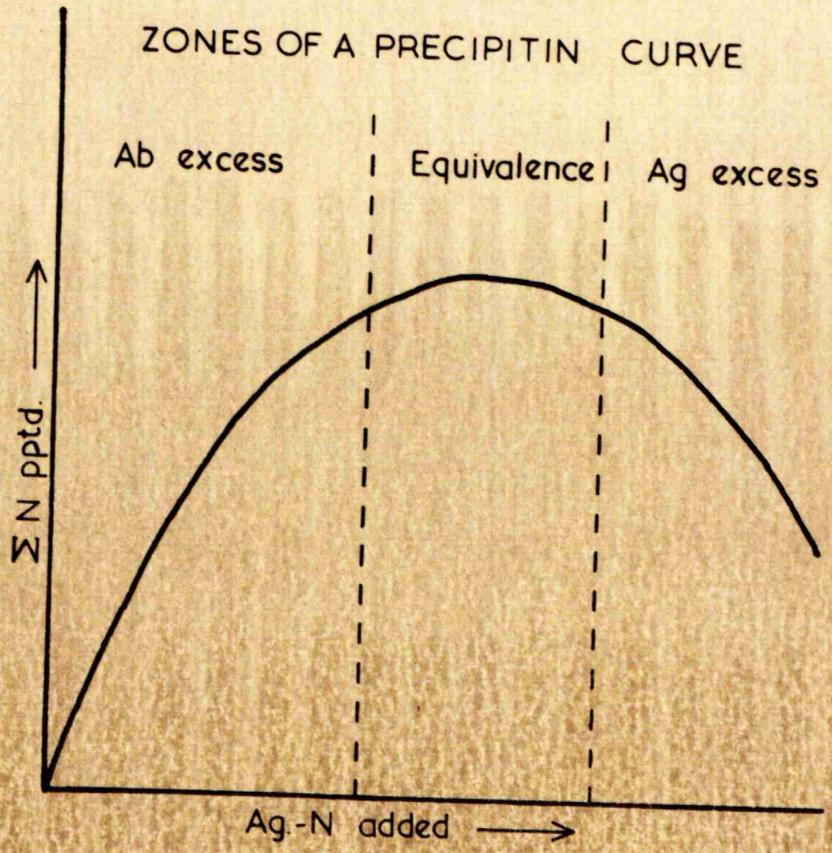

Fig. 6. Typical mammalian precipitin curve showing the three characteristic zones

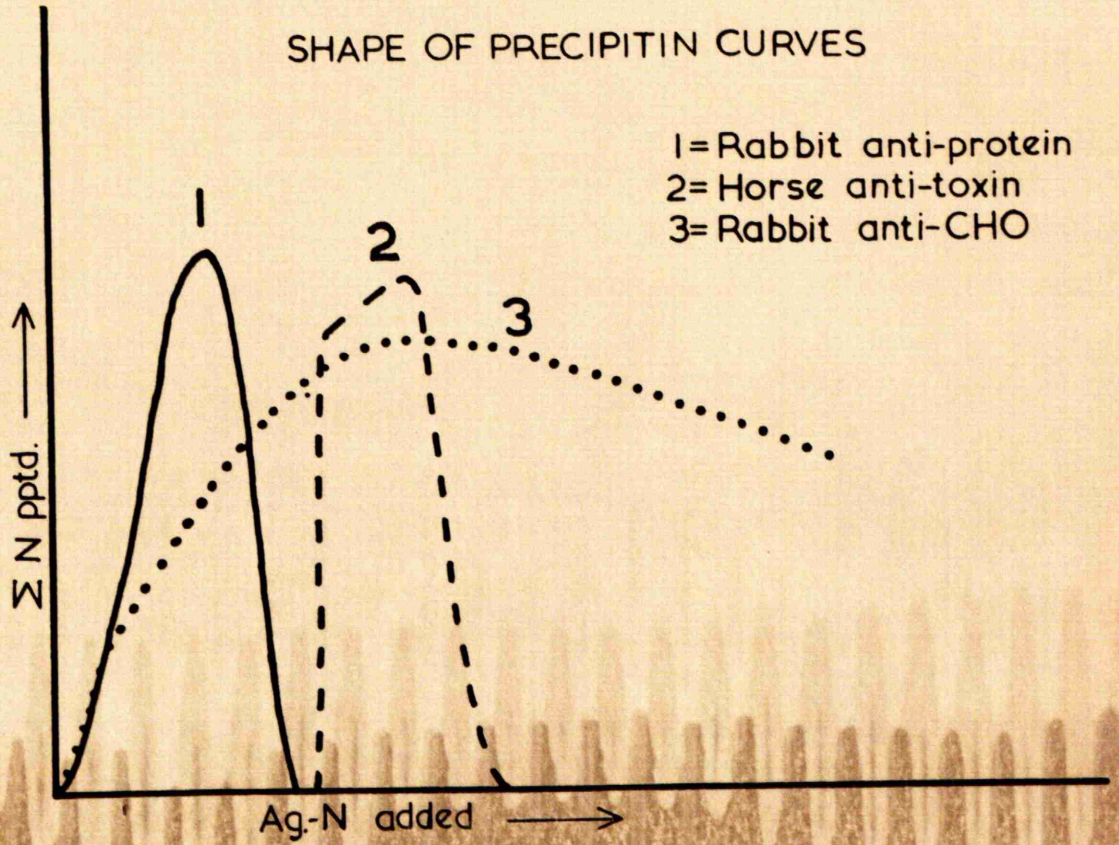

Fig. 7. *The three principal types of precipitin curve in mammalian systems*

3. The rabbit anti-carbohydrate type of precipitin curve differs from the previous two in possessing a broad region of maximal precipitation and in being less susceptible to inhibition in excess antigen.

Both chicken anti-protein systems yielded precipitin curves that presented the three zones found in mammalian systems (cf. Figs. 4 and 5), but the overall patterns most closely resembled the anti-carbohydrate curve of the rabbit and horse because of the comparatively broad plateau of maximum precipitation that was relatively unaffected by moderate antigen excess (N.B. in Figs. 4 & 5 the antigen added scale is logarithmic). Only when considerable excess of antigen was present did inhibition of precipitation make itself apparent. Although the anti-BSA system appeared the more readily inhibited, the degree of inhibition in both systems was much less marked than in the case of the rabbit.

It is true that an extended plateau of maximum precipitation can result from the use of a complex mixture of antigens producing several different equivalence points in the same region, (Cushing & Campbell, 1957, p. 288) but in these experiments the antigens BGG and BSA were of a high degree of purity (> 97% and 99% respectively by electrophoresis), so that such contaminants as were present should not have been capable, on the basis of a single intravenous injection, of exerting an antigenic influence comparable to that of the principal components. On the other hand, the work of Cohn and associates (Cohn, Wetter & Deutsch, 1949; Wetter, Cohn & Deutsch, 1952) has served to show that antigens known to contain very small amounts of antigenically active contaminants can stimulate the production of antisera containing disproportionately large amounts of antibody to the minor components, and the possibility of such a mechanism operating

in the chicken anti-BGG and anti-BSA systems must be kept in mind. This matter is touched on in more detail in the discussion concerning the specificity of precipitation in varying concentrations of NaCl (pp 122 to 125).

None-the-less, the type of precipitin curve having a reasonably well-defined but rather broad region of maximal precipitation would appear to be characteristic for the anti-protein system in the fowl, for similar curves have been derived from the work of Wolfe and associates (e.g. Gengozian & Wolfe, 1957).

Equivalence zone and maximal precipitation:

"Zone of equivalence", as defined by Heidelberger (see Kabat & Mayer 1948, p.23) refers to that part of the curve where neither antigen nor antibody, or only traces of one or other, are present in the supernatants of specific precipitates; it often coincides with, or is close to, the point of maximal precipitation. On the assumption of complete precipitation of added antigen in the zone of antibody excess, Heidelberger and Kendall devised their method of quantitative precipitin analysis. Supernatants of successive tubes were tested for residual antigen, until the first trace was detected. Subtraction of added antigen N added from total N of precipitates gave the amount of antibody N precipitated up to the equivalence zone. Whereas the Heidelberger and Kendall method worked satisfactorily with the rabbit anti-ovalbumin type of precipitin curve, it is not applicable to chicken anti-protein systems for the simple reason that there is no analogous "equivalence point" in the chicken precipitin curves. By reference to Figs. 4 and 5 it can be seen that although there is practically 100% precipitation of added antigen in the region of extreme antibody

excess, the amount begins to fall quite steeply before maximum antibody precipitation has been reached. In consequence there is no point at which complete precipitation of both antibody and antigen occurs simultaneously and the classical method of determining equivalence fails to indicate the complete precipitin content of a chicken antiserum.

That maximal precipitation of antibody did not occur until conditions of moderate antigen excess were operative implied the simultaneous presence in the supernatant of precipitating antigen and unprecipitated antibody. There are two possible explanations for the presence of both antigen and antibody in the same supernatant. Either the antigen and antibody so present were in the form of combined but soluble antigen-antibody aggregates that required a higher antigen content for precipitation, or there was present in the antigen used for immunisation a significant amount of impurity that had stimulated an individual precipitin response.

Since the proportion of impurities in the relatively pure samples of BSA and BGG used must have been small, it would have needed a considerable amount of the whole antigen in the precipitin test to make apparent the influence of the impurity which was consistent with the observed facts in both chicken precipitin systems.

According to Cohn (1952,pp. 308 et seq.) the presence of such an immunologically active minor component in the antigen employed in immunisation and in conducting the quantitative test, would explain the lack of a well-defined equivalence zone, for when the major component was at equivalence there would be in the supernatant unprecipitated antibody to the minor component, so that tests on the supernatant would show an antibody excess. The further addition of total antigen would result in an antigen excess of the major component and

T A B L E 7.

Composition of precipitates obtained in anti-BSA systems

Conditions	*Chicken*	*Rabbit*
Ab excess	Ab_9Ag	Ab_6Ag
Max. Pptn	Ab_9Ag_2	Ab_7Ag_2
Ag excess	Ab_3Ag	Ab_2Ag

Comparison of chicken and rabbit anti-serum albumin systems

Rabbit data taken from Heidelberger (1956) p. 47.

an antibody excess of the minor component. "Thus the clearest evidence for more than one reacting substance is the presence of both antigen and antibody in any given supernatant."

Antibody N/antigen N ratios: In both the BGG and BSA systems, the composition of the precipitate varied with the different proportions of antigen and antiserum used in the tests. This is illustrated in Figs. 8 and 9, in which AbN/Ag N ratios are plotted against their respective precipitin curves. The type of curve described by the N ratios in both chicken systems conforms to the pattern observed in precipitates obtained from rabbit anti-protein sera (Cushing & Campbell, 1957, p.282). In the region of extreme antibody excess an antibody-rich precipitate was obtained but the N ratio was soon reduced as more antigen was made available for combination with antibody. Throughout the zone of maximal precipitation and in the region of excess antigen the fall in the N ratio continued but was much less pronounced.

For the BSA system, the N ratios varied from about 20 in extreme antibody excess to 6.9 at a point in considerable antigen excess. Assuming molecular weights of 70,000 and 160,000 respectively for BSA and chicken antibody the molecular ratios were found to range from 8.4 where there was an abundance of antibody, to 3 in the zone of partial inhibition due to excess antigen, with a ratio of 4.5 obtaining at maximal precipitation.

From this data can be calculated an empirical composition for precipitates formed under the different conditions existing in the three principal zones of the curve. The composition of precipitates is indicated in Table 7. and is there compared with similar composition data taken from a rabbit

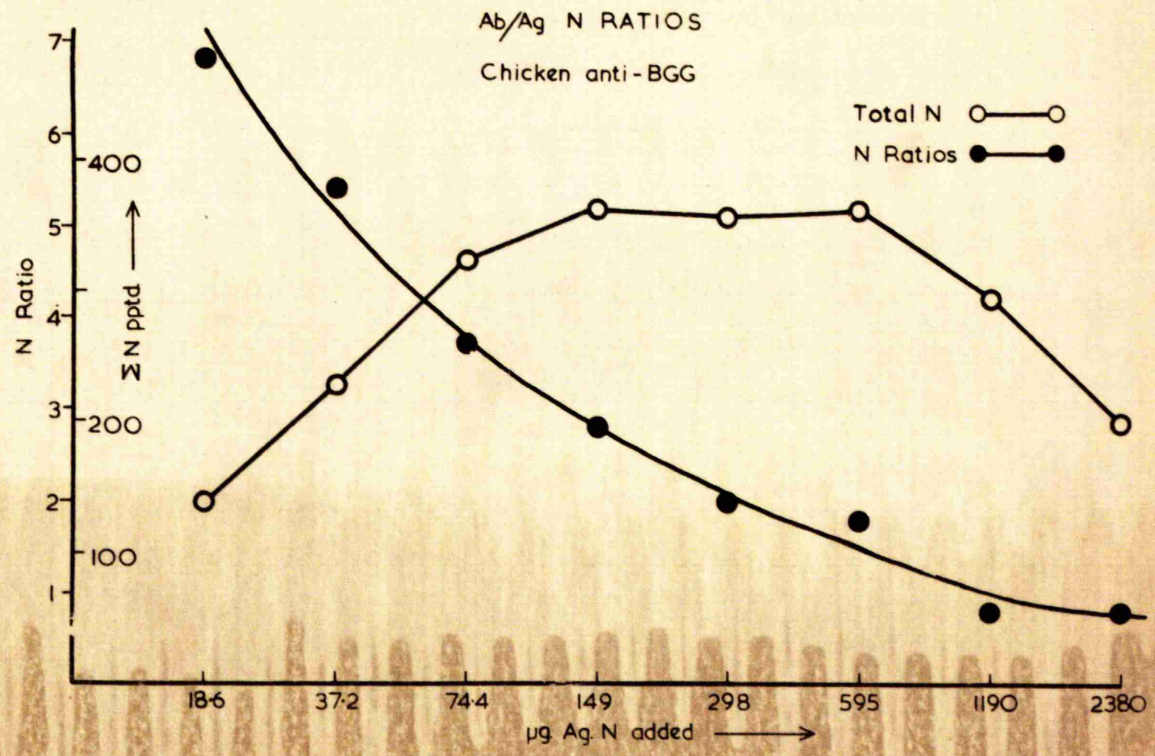

Fig. 8. *The chicken anti-BGG system showing the ratio of antibody N to antigen N at various points along the precipitin curve*

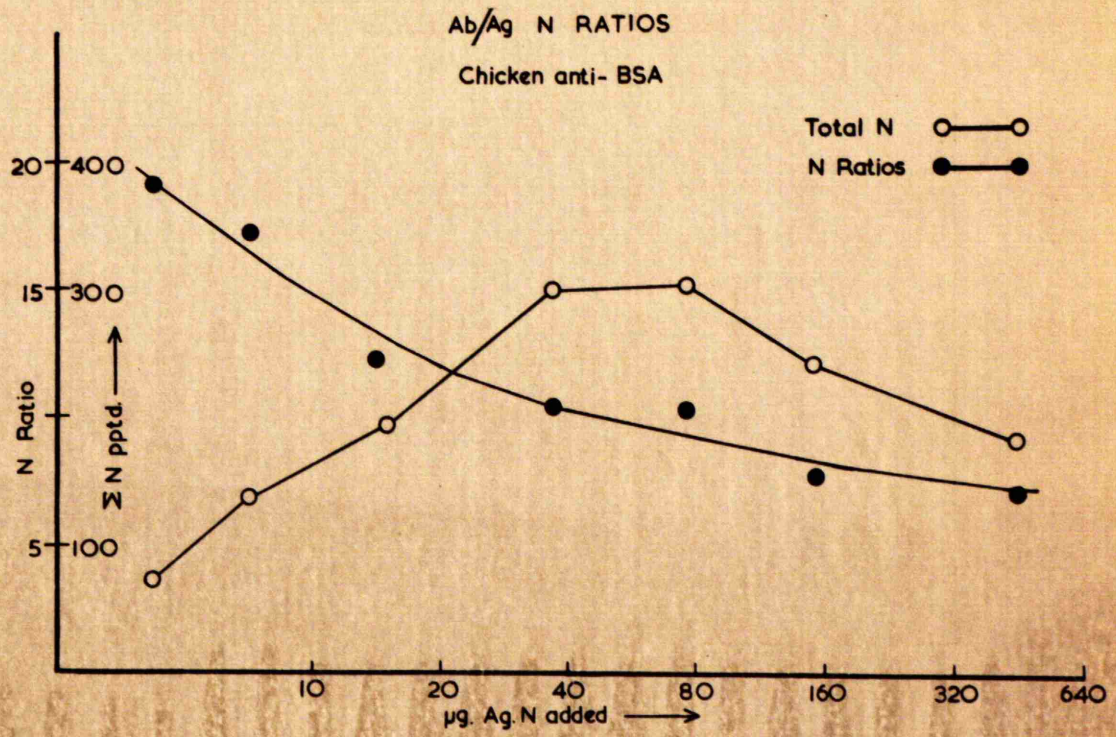

Fig. 9. *The chicken anti-BSA system showing the ratio of antibody N to antigen N at various points along the precipitin curve*

anti-serum albumin system described by Heidelberger (1956 p. 47). A good correlation exists between the precipitate composition formulae for the two species, so that in this respect there would not appear to be any outstanding difference in the nature of the precipitate.

With the majority of antigens, the precipitate contains more antibody than antigen but because of the solubilising effect of excess antigen, the Ab/Ag molecular ratio never falls below a certain limiting value, this value representing the approximate composition of the compound having the smallest proportion of antibody that will still precipitate. When the antigen molecule is small, as in the case of ovalbumin or serum albumin, the smallest Ab/Ag molecular ratio obtainable is about 2. Antigens of higher molecular weight require a greater number of antibody molecules to precipitate one molecule of antigen, e.g. viviparus haemocyanin with a M.W. of 6.5×10^6 needs about 35 rabbit antibody molecules to effect precipitation in antigen excess (Boyd, 1956, p. 316).

The AbN/AgN ratios in the chicken anti-BGG system ranged from 6.8 in antibody excess to a value just less than 1 under conditions of excess antigen. The lower N ratio figures for this system compared to the anti-BSA system are probably attributable to the higher molecular weight of the antigen. Molecular ratios were only slightly lower than the N ratios because the molecular weights of antibody and antigen are almost identical (160,000 and 180,000 respectively).

Added note: Since this work was completed there has appeared a very recent paper by Orlans, Rose and Marrack (Immunology, 4, No. 3, p. 262, July 1961) in which the

authors present evidence for the existence in chicken anti-BSA sera of two types of homologous antibodies having molecular weights of 180,000 and 600,000 respectively. Both 'light' and 'heavy' antibodies participate in the precipitin reaction performed in 8% NaCl but it is not yet known in what relative proportions they occur in precipitates formed at different points in the curve. Clearly the existence of two types of chicken antibodies of widely different molecular weights renders invalid the empirical antibody-antigen molecular ratios derived for precipitates analysed in the BSA and BGG systems. For the determination of more accurate ratios it will be necessary to know more about the relative combining properties of 'light' and 'heavy' antibody in the different zones of the precipitin curve.

The amount of antigen precipitated: When the amount of antigen that was precipitated at any one point was expressed as a percentage of the total amount of antigen added at that point, the curve obtained by plotting percentage values across the precipitin curve revealed interesting information. Although there was virtually 100% precipitation of antigen in extreme antibody excess the trend was not continued up to the point of maximum precipitation; there was instead a rather sudden fall that was maintained into considerable antigen excess. Such a significant change in the percentage of antigen precipitated throughout the relatively broad region of maximal precipitation of antibody produced the type of curve reminiscent of the one obtained in complement titration and suggested a method for the rapid analysis of the precipitating ability of an antiserum. A description of this method follows.

4. *A RAPID METHOD FOR THE DETERMINATION OF THE PRECIPITIN CONTENT OF AN ANTISERUM, USING TRACE-LABELLED ANTIGEN*

In any precipitating system the percentage of added antigen involved in the precipitation of the maximum amount of antibody provides a measurable end-point for the precipitating ability of the serum, an end-point that is particularly sensitive because of the degree of change occurring in percentage values over the region of maximum antibody precipitation (cf. Fig. 4 and 5). The AbN/AgN ratio over the maximum antibody range having been predetermined, it is a matter of simple proportion to calculate the amount of antibody at the corresponding point in terms of N content, and thus to obtain a figure for the antibody concentration of the antiserum.

Such a titration system has the further advantage that any percentage determination falling well outside the end-point region can still yield useful information, for the position on the titration curve can be readily evaluated by inspection and a more appropriate amount of antigen selected for the second titration of the same serum.

For example, if an initial titration indicated 100% precipitation of added antigen, conditions of extreme antibody excess would obviously have been operating, and for the second titration a greater amount of antigen would be necessary. Conversely, where too great an excess of antigen prevailed, only 10 - 20% of antigen would be precipitated and the next test would require less antigen. Within the limits of the two extremes of antibody excess and antigen excess, where the curve described by the percentage of antigen precipitated is straight, it is possible to determine with reasonable accuracy the actual amount of antigen which would require to be added to the antiserum to permit of titration to the end-point.

When dealing with a large number of sera in a comparative experiment, the advantages of such a 'screening' system are obvious. By means of a single titration using a given amount of antigen, the sera can immediately be divided into at least three classes - strong, measurable and weak - after which each class can be more extensively examined using the appropriate quantities of antigen.

Determination of the percentage of antigen precipitated eliminates the need for analysis of precipitates, for, when a radioactive label is attached to the antigen, measurement of residual antigen in supernatants of specific precipitates is more convenient, and can be rapidly and accurately performed in a well-type scintillation counter. Use of supernatants in this fashion avoids washing, handling and analysis of the specific precipitates, an obvious merit when such precipitates are themselves small, e.g. when analysing low titred sera.

The utility and expediency of the determination of a fixed end-point in a given precipitating system as a measure of the precipitin content of an antiserum seemed particularly suited to the type of experiments planned for investigating immune unresponsiveness in the chicken. It provided a method for the rapid comparative measurement of a biological function of antisera, (viz. ability to precipitate antigen) and did not make heavy demands on serum for analysis, a point of particular importance when the serum has to be obtained from very young animals with small blood volumes.

Perusal of the appropriate literature revealed that the method was not original, for the same principle lay behind the scheme devised by Eisen and Keston (1949) for

studying a rabbit anti-BSA system. As their end-point, they chose the first indication of a rise in the amount of free antigen in the supernatants: in other words, they were determining the antigen excess end of the equivalence zone. The accuracy and utility of the technique were more extensively investigated by Talmage and Maurer (1953) in a number of rabbit anti-protein systems. They pointed out that the turning of the 'precipitate fraction curve' (i.e. the curve describing the percentage of antigen precipitated) as it approaches 100% antigen precipitation, precludes precise determination of the first trace of antigen in supernatants, and that antigen precipitation is probably never 100% complete. They therefore recommended as an end-point, the point of 80% antigen precipitation (P. 80) which lay on the straight portion of the precipitate fraction curve and was thus capable of quite accurate determination if values above and below the point were known. (The analogy to the 50% unit of complement activity was drawn by these authors). Very good correlation was found when the antigen precipitating ability of antisera to serum protein antigens determined by the P 80 test, was compared with that obtained by N analysis of the specific precipitates.

Both groups of workers appreciated the rapidity and simplicity of labelled antigen analysis and were impressed by the sensivity of the method. However, as in all precipitin systems, a limit to sensivity is imposed by the solubility of the antigen-antibody complex. Of particular advantage was the small amount of serum required from individual animals for complete and-point titration.

A further adaptation of the test was reported by Dixon, Maurer and Deichmiller (1954) in a paper describing

the primary and anamnestic responses to serum proteins in the rabbit. As previously, P 80 was chosen as the end-point, but with the aid of AbN/AgN ratios determined at P 80 for each system they calculated the antibody content of the sera as explained above and were able to express results in μg antibody N per ml of serum.

Elsewhere in this thesis are described experiments in which BSA was the antigen. In many cases, serological analyses were performed by means of titration of precipitated antigen. The quantitative precipitin study on the chicken anti-BSA system showed that maximum antibody precipitation occurs when 50% of the added antigen has been precipitated, and that the AbN/AgN ratio at this point has a value of 10. After titrating to the end point of 50% antigen precipitation (P 50), the actual amount of antigen precipitated, in terms of μgN, was multiplied by a factor of 10, and the resultant figure expressed as μg of antibody N per unit volume of serum used in the test. Although such a treatment is open to criticism on the grounds that the AbN/AgN ratio is subject to some degree of animal to animal variation (Talmage & Maurer, 1953), it should be noted that the N ratio figure of 10 was derived from a quantitative test performed on a pool of antiserum from several young birds of the same cross used in all later experiments. Further, the significance of the results of these experiments lies in the comparative antibody concentrations expressed and not on their absolute values. Any error therefore, is one of expression, not one of fact.

5. *THE SPECIFICITY OF IMMUNE PRECIPITATES FORMED IN HIGH CONCENTRATIONS OF NaCl.*

In the course of several experiments designed to investigate the effect of varying concentrations of NaCl on precipitation in chicken immune sera Goodman, Wolfe and Norton (1951) carried out qualitative and quantitative tests on a number of pooled samples of chicken antiserum at increasing NaCl concentrations of 1, 2, 4 and 8%. With each increase in salt concentration they obtained a marked rise in the amount of total N precipitated from the system. This effect was thought to be due either to the participation of some non-specific reaction or to more complete antigen-antibody precipitation, and attempts were made to determine which of the two factors was involved. The following is a summary of their principal observations.

The increase in N precipitation affected the antigen excess zone only, and a further extension of this zone could be achieved by raising the NaCl concentration above 8%. When the supernatants of precipitates formed in low salt concentration were raised to 8% NaCl additional precipitation occurred and free antibody was detectable. The AbN/AgN ratios at the point of maximal precipitation were never greater in 8% NaCl than in lower salt concentration. After fractionation of chicken antiserum into albumin, water soluble globulin (pseudoglobulin) and water insoluble globulin (euglobulin), the three fractions were brought to the volume of the original serum and tested with antigen. Antibody activity could be detected only in the euglobulin fraction, which precipitated to a greater degree in 8% NaCl than in lower concentrations. In one case the reconstituted euglobulin fraction had an antibody content equivalent to that of whole serum, and in another case a slightly decreased precipitation was obtained.

On the basis of the above described findings Goodman et al. (1951) concluded that the increased N precipitation in reaction media containing more than 1% NaCl was due, not to coprecipitation of Non-specific N, but to a more complete precipitation of the antigen-antibody complex. Their experiences in handling chicken antisera led them to advocate that in order to achieve maximal precipitation of chicken precipitins all quantitative tests should be performed in a medium of at least 8% NaCl. An exact reason for the incompleteness of antigen-antibody precipitation in low NaCl concentration was not given, but possible explanations were suggested. These will be described and discussed later (see p. 118).

The only other available information of a quantitative nature concerning chicken precipitating antisera was that of Deutsch, Nichol and Cohn (1949). Their evidence indicated that, in a chicken anti-protein system which they tested at 0.9% NaCl, up to 50%, of the immune precipitate appeared to be α globulin. These workers made no reference to the behaviour of chicken antiserum in higher salt concentrations, but on the basis of their immunochemical and electrophoretic studies it was recommended that for quantitative work fractionation of serum was essential, in order to eliminate non-specific coprecipitation of the globulins (Cohn, 1952).

In view of these controversial findings relating to the specificity of precipitates obtained from chicken immune serum it was considered desirable to repeat certain aspects of Wolfe's work relating to increased N precipitation in higher concentrations of NaCl, paying particular attention to analyses of precipitates. A method employing

isotopically trace-labelled antigen was particularly suited to the pursuit of this investigation requiring as it did sensitive and accurate determinations of the composition of the precipitate.

Antiserum

Immune serum was obtained by immunising eleven young adult birds (eight males and three females) with Armour's BSA. Each bird received a single intravenous injection of 1.0 ml volume containing 47.5 mg protein dissolved in 0.15 M-NaCl, regardless of body weight. Seven days later, each bird was blood sampled and the serum used for a qualitative precipitin test. All birds having responded well to the antigenic stimulus, they were exsanguinated on the eighth day and the sera held at - 20°C until required for use.

Antigen

For the quantitative test ^{131}I-trace-labelled BSA was prepared by the standard iodine-iodide exchange method. Protein N concentration was determined by Kjeldahl analysis and was correlated with radioactivity of the labelled antigen. Serial dilutions of antigen were prepared from standard stock solutions and confirmatory N analyses performed on some of these dilutions. Commencing with the most dilute solution of antigen used, these antigen preparations were numbered Ag_1, Ag_2 ... as listed below.

Antigen number	µg N /ml
Ag_1	3.82
Ag_2	7.64
Ag_3	15.28
Ag_4	38.2
Ag_5	76.4
Ag_6	152.8
Ag_7	477.5
Ag_8	624.0

Versene

To prevent the coprecipitation of complement N during specific precipitation, the disodium of salt of versene was incorporated into the test. 3.723 g of sodium versenate were dissolved in 500 ml of a phosphate buffer of pH 7.4 and to this was added 500 ml of 1.8% NaCl. As a slight fall in pH was effected it was necessary to add a few drops of 2N-NaOH to restore pH to 7.4. The final solution was 0.01M with respect to versene and 0.9% with respect of NaCl.

Compensating Saline Solutions

The three reagents described above, antiserum, antigen and versene, were prepared in a NaCl concentration of 0.9% and in order that the final saline concentration in two of the tests could be raised to 4% and 8%, compensating solutions of 13.3% and 29.3% respectively were prepared. For the 1% saline concentration, 0.9% NaCl was used throughout.

The Quantitative Test

For reference purposes the three NaCl concentrations at which the test was to be conducted were designated A, B, and C for 1%, 4% and 8% respectively. Triplicate tubes were set at every antigen dilution for each of the three series, with the exception of Ag_8 at which point only the C series was tested. As antigen controls every antigen dilution in each series incorporated a tube containing normal chicken serum in place of immune serum. Serum control tubes, in triplicate for each series, contained immune serum, versene, and 0.9% NaCl in place of antigen. The design of the quantitative test is illustrated on the next page.

The quantitative test

Immune serum	*Ag number* 1	2	3	4	5	6	7	8	*Serum control*
Triplicate samples 1									*Immune serum, Versene, & Saline*
2									
3									
Ag control (NCS)									

To every tube were added the following four reactants, in 1.0 ml volume, control tubes being suitably modified.

(i) serum
(ii) versene
(iii) antigen
(iv) compensating NaCl solution

These components were added to the tubes in the order stated, (i) & (ii) being well mixed and allowed to stand at room temperature for 5 - 10 minutes prior to addition of (iii) and (iv) allowing versene to exert its full chelating effect on serum Ca^{++} and Mg^{++} before the third component was added. During the addition of compensating salt solutions the tubes were agitated constantly to ensure a rapid complete mixing and dilution of the incoming saline, to avoid any salting-out effect resulting from local high NaCl concentration in the tube. When all four components had been added, the tubes were sealed with 'parafilm' and thoroughly shaken to mix the contents. Following a 60 minute period of warm incubation in a water bath at 37°C, all tubes were transferred to the refrigerator (4°C) for overnight storage.

The following day, the standard procedures for the various analyses were carried out. After an initial 45 minute period of centrifugation at 1500 g the precipitates were twice washed, each time with 0.5 ml ice-cold NaCl of appropriate strength and re-centrifuged after each washing. The washings were added to the original supernatants and the whole made up to a standard volume of 10 or 25 ml with dilute NaOH. To each precipitate was added 10 ml of 0.1N NaOH and then the tubes were incubated at 37°C for 30 minutes to obtain complete solution of the precipitates. Subsequent to these treatments all tubes were sealed with 'parafilm' and stored in the refrigerator until such time as they were required for analysis.

Analytical Procedures

Total N analyses by the standard Markham modification of the micro Kjeldahl procedure were carried out on aliquots of total precipitates. All radioactivity determinations were made in a scintillation counter, (annular ring crystal), linked to a Panax 100C scaler. The antigen N content of the dissolved precipitates was determined by correlation of direct radioactivity measurements on the whole sample, or on aliquot of the total volume, with the specific activity of a standard solution prepared from the trace-labelled antigen.

Supernatants of the precipitates were similarly analysed to confirm that no loss of antigen was involved in washing the precipitates.

Results

From the analyses were obtained three sets of essential figures:

TABLE 8.

Mean experimental results

Chicken anti-BSA quantitative precipitin tests

All values refer to µg N

Ag N added	Total N pptd. A	B	C	Ag N pptd A	B	C	Ab N pptd A	B	C
3.8	57	86	74	3.0	3.6	3.7	54	83	71
7.6	86	146	138	5.6	7.0	7.6	81	139	131
15.3	107	220	194	8.8	13.7	14.7	98	207	179
38.2	62	245	298	4.1	19.7	26.2	58	226	272
76.4	42	174	302	1.7	15.9	26.7	40	158	275
153	24	95	240	0.7	10.0	27.6	23	85	212
478	11	32	182	0.5	2.0	23.2	11	30	159
624			185			20.6			164

All figures represent the means of triplicate samples, minus the appropriate 'blank'. A, B, and C refer to NaCl concentrations of 1, 4, and 8% respectively.

(i) total N precipitated
(ii) Ag N in the precipitates.
(iii) Ab N in the precipitates.

These are presented in Table 8.

Antibody N represents the difference between total N and Ag N in the precipitates.

Some comments on the figures in Table 8 are necessary. Although only one set of figures is presented for each amount of antigen used, in all cases triplicate results were obtained. A mean was calculated and from each mean value was subtracted any necessary background indicated by control results.

Triplicate values for antigen N precipitated were extremely close in every case and the background of non-specific precipitation was very low. Correlation between triplicates in total N determination was always reasonable except for a few instances where one of the three results, being either greater or smaller than the two in agreement, produced some irregularity. In every case, however, a true mean was calculated and from this figure was subtracted the appropriate background of non-specific N precipitated in control tubes containing immune serum and saline. The values obtained for the amounts of non-specific N precipitated in these control tubes were 7.4, 4.7 and 12.7 μg N respectively, in the 1, 4 and 8% NaCl series.

The composition of the precipitates having been determined by total N and radioactivity analysis, two other sets of data of significance were available. These were

(i) the Ab N/Ag N ratios in the precipitates,
(ii) the percentage of added antigen actually precipitated at each point.

TABLE 9.

AbN/AgN ratios and % Ag precipitated in the Chicken-anti-BSA quantitative precipitin test.

µg Ag N added	AbN/Ag N ratios			% Ag pptd.		
	A	B	C	A	B	C
3.8	18.1	23.0	19.1	79.2	95.4	95.5
7.6	14.4	19.8	17.2	73.4	91.9	99.7
15.3	11.1	15.1	12.2	57.8	89.8	86.0
38.2	14.1	11.5	10.4	10.8	52.0	68.6
76.4	23.6	9.9	10.3	2.2	21.0	35.0
153	32.9	8.5	7.7	0.5	6.6	18.1
478	29.9	15.2	6.9	0.1	0.5	6.1
624			8.0			3.3

These figures for Ab N/Ag N ratios and for % Ag precipitated were derived from the values given in Table 8

This information is contained in Table 9.

In Figures 10, 11, 12, some aspects of the accumulated data are presented graphically.

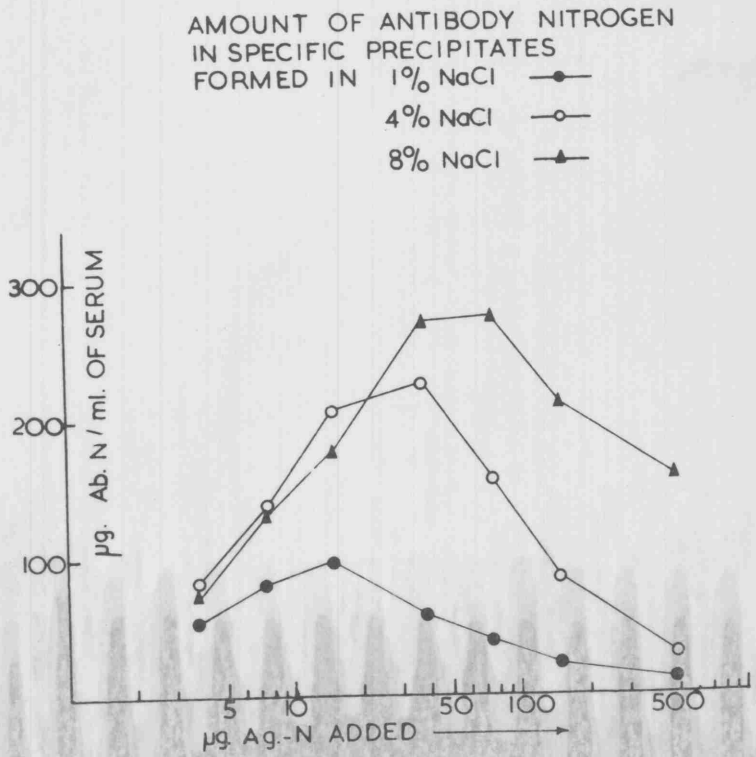

Fig. 10. *Note that with each increment in NaCl concentration a greater amount of antibody N is precipitated*

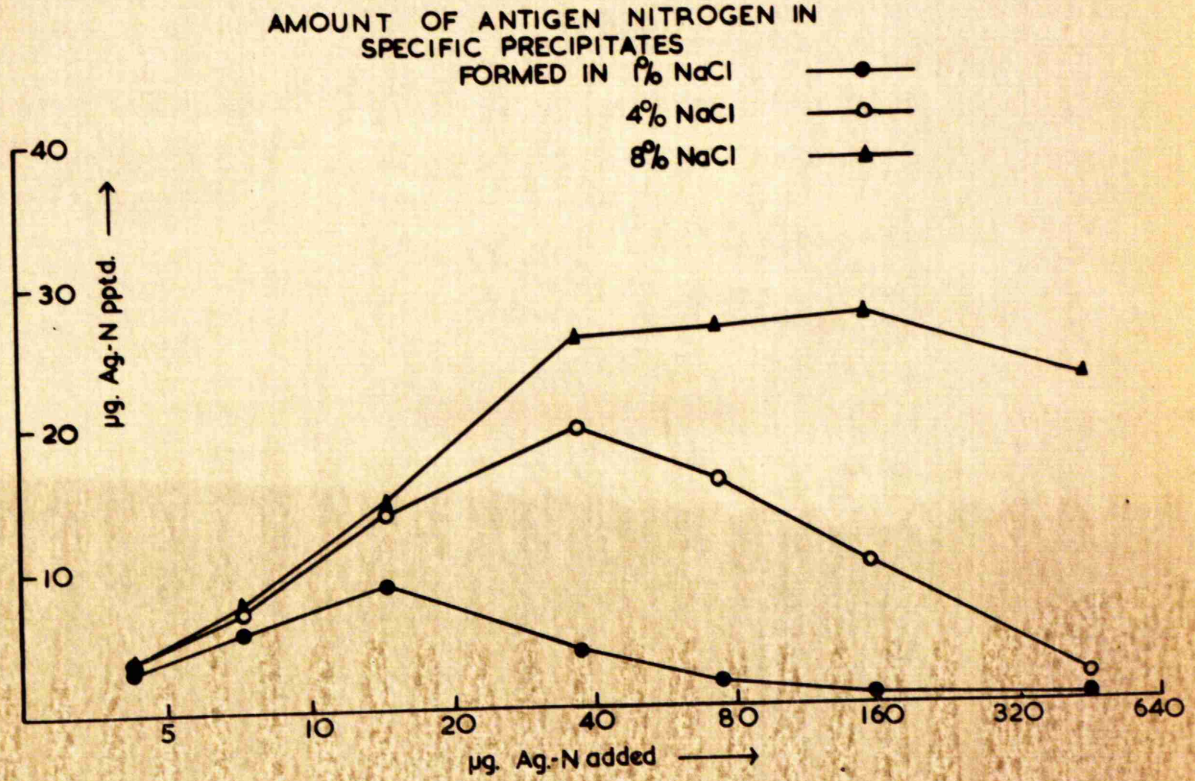

Fig. 11. *The amount of antigen N precipitated is greater in higher concentrations of NaCl*

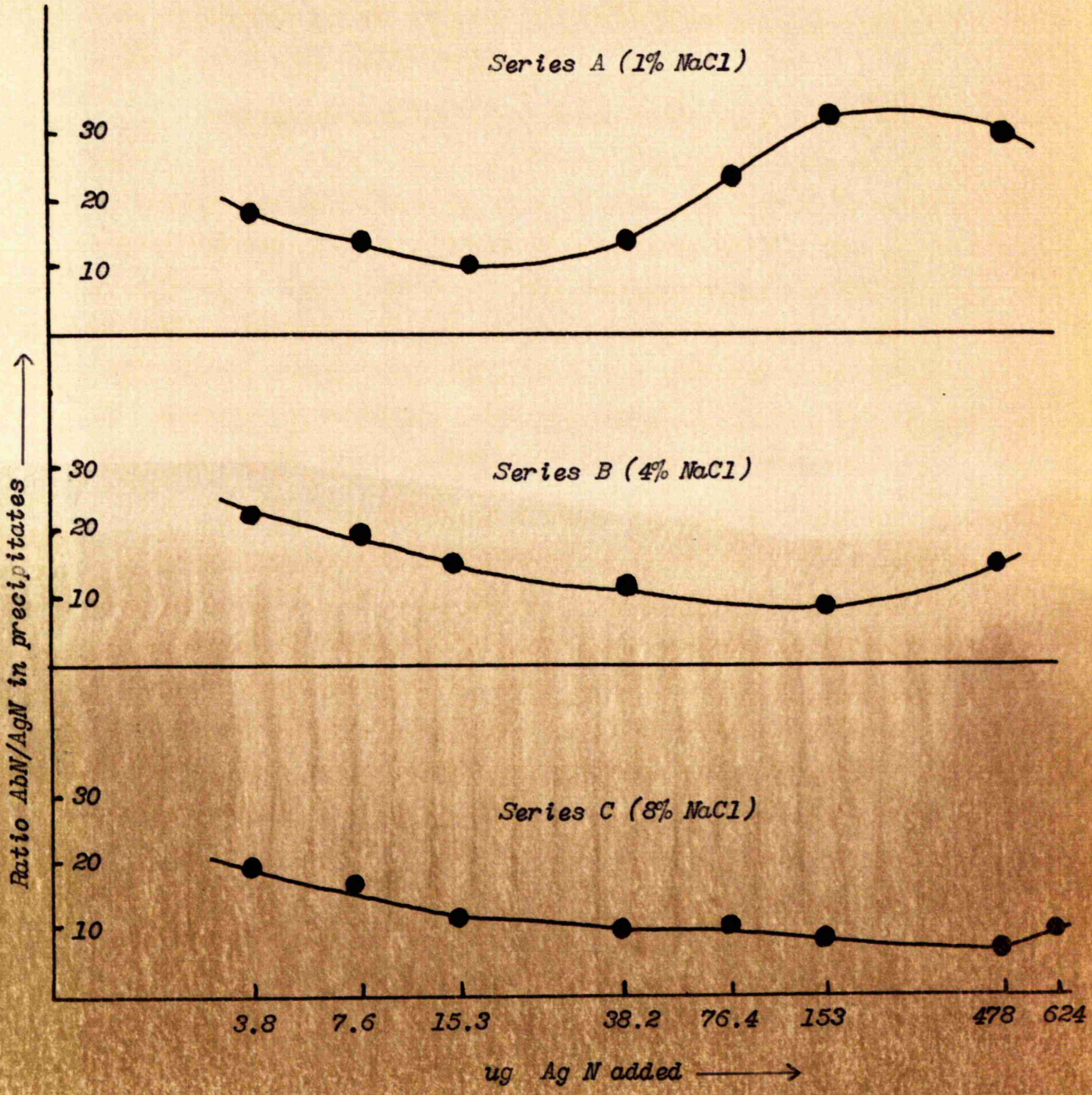

Fig. 12. AbN/AgN ratios in precipitates formed in 1%, 4% and 8% NaCl with the chicken anti-BSA system.

Discussion

The effect of increased NaCl concentration on the amount and specificity of precipitates.

From the tabulated data and associated figures it is obvious that with each increase in NaCl concentration there occurred an increase in the amount of total N precipitated, the increase taking place as progressively greater amounts of antigen were added to the antiserum. Further, by virtue of the sensitive index provided by the trace label, it was made quite clear that there was a concomitant increase in the amount of antigen precipitated (Figure 11), which suggested an increase in specific precipitation.

A fairly stringent test of the nature of increased N precipitation is available in the analysis of comparative AbN/AgN ratios at the point of maximum precipitation. Under conditions of more efficient precipitation of antigen-antibody complexes the ratio should undergo no increase whereas it would naturally rise if the additional N were derived from non-specific sources. When Goodman et al. (1951) applied such a test to their results they obtained figures of 9.6, 7.7 and 7.3 for the AbN/AgN ratios at maximal precipitation in 1, 4 and 8% NaCl respectively. In the study described here, in which trace labelling of the antigen with ^{131}I made it possible to detect minute quantities of the antigen with great accuracy, the corresponding figures obtained were 11.1, 11.5 and 10.3 in 1,4 and 8% NaCl. These figures are higher than those of Goodman et al, but the analytical technique which these workers employed in estimating non-precipitated antigen was such that it did not permit of very accurate determinations. It is noteworthy that in a later study, in which a more sensitive method was

used to determine antigen, N ratios of 10-11 were obtained (Gengozian & Wolfe, 1956).

Thus the results of this quantitative study of the precipitin curves described by the chicken anti-BSA system in media of 1, 4 and 8% NaCl provided confirmation of the observations of Goodman and co-workers relating to the considerable increment in total N of the precipitate when the quantitative precipitin test is conducted in a saline medium of greater than 1%. Further, it provided corroboration of their view that the increased precipitation is of a specific nature.

Although Deutsch et al. (1949) implicated α globulin in chicken precipitin systems as a non-specific nitrogenous component that was capable of providing up to 50% of the N found in immune precipitates, no evidence of non-specific precipitation was obtained in this experiment with trace-labelled antigen. Failure to demonstrate co-precipitation of α-globulins has been reported by a number of workers (Goodman and Ramsay 1957, Banovitz, Singer & Wolfe, 1959, Banovitz & Wolfe 1959, Makinodan, Gengozian & Canning, 1960).

The considerable amount of non-specific N in immune precipitates described by Deutsch et al. may have been the result of differences in the method of production of immune serum for these workers used serial intraperitoneal injections of antigen compared to the more usual schedule of single or double doses of antigen by the intravenous route. Furthermore, all tests were performed in 0.9% NaCl and no reference was made to ageing of serum prior to testing.

More recently, Makinodan et al. (1960), using ultracentrifugal and immunoelectrophoretic analytical techniques, have demonstrated that a normal serum component can

co-precipitate with the antigen-antibody complex in the chicken anti-BSA system throughout the entire precipitin curve, and can be responsible for 20% of the recorded antibody N at equivalence. The coprecipitating factor was identified as macroglobulin of β or γ type, and was present in normal and immune sera. It is considered to be analogous to the complement N factor, properdin and the rheumatoid factor, but is still of unknown biological significance.

Having shown that raising the concentration of NaCl in the test medium markedly increased the amount of N precipitated from chicken antiserum, and having established that such increase was the result of more complete precipitation of antigen-antibody complexes, it was necessary to consider what mechanism might be involved. This problem was first tackled by Goodman et al. (1951), who suggested two possible explanations.

(i) From antibody excess to antigen excess antigen-antibody complexes of increasing antigen content and increasing solubility are formed. Increasing the NaCl concentration serves to reduce the solubility of these compounds.

(ii) Antigen-antibody compounds dissociate readily in media of low ionic strength and the effect of raising the NaCl concentration is thus to reduce the degree of dissociation and to promote precipitation.

Avian globulins are more sensitive to the salting-out effect of NaCl than are mammalian globulins. Goodman and Wolfe (1952) noted that precipitation of chicken, owl and pheasant globulins commenced at a NaCl concentration of 14% and that antibody was present in the globulins precipitated at 19% NaCl. On the other hand it took 27-31% of NaCl to bring about any salting-out of rabbit globulin. A salting

out of soluble antigen-antibody complexes was thus considered as a possible explanation for the observed facts.

The comparative electrophoretic studies described by Cohn (1952) are significant to this discussion. Whereas the serum antibody response of the rabbit is largely in the γ_2 globulin fraction, chicken antiserum appears to lack a component corresponding in mobility to mammalian γ_2 globulin. There are, however, two distinct fractions in the γ globulin region, both possessing considerably higher mobilities than their mammalian counterparts, and thus carrying a higher net charge. It would seem reasonable to assume therefore, that the role of NaCl in chicken precipitating systems is to effect a neutralisation of charge and to promote more complete precipitation of formed antigen-antibody complexes.

The matter was taken further by Goodman, Wolfe and Goldberg (1954) who investigated the effect of variation in ionic species and concentration on precipitate formation in chicken systems. Their findings indicated that the pronounced effect of electrolytes on the chicken precipitin system was the outcome of a moderate degree of anion binding by the immune aggregate, the greatest amount of precipitation occurring at high ionic strengths, suggesting that a salting-out of soluble antigen-antibody aggregates was important in obtaining complete precipitation. Banovitz et al. (1960) found that in 1% NaCl there was a preferential precipitation of the antibody component that had the lower electrophoretic mobility, and therefore the lower net charge, while the faster antibody component, with the higher net charge, was concentrated in the fraction precipitated in 8% NaCl. Such evidence lends support to the view that a higher NaCl concentration promoted a more efficient precipitation of antigen-antibody complexes by virtue of a salting-out effect.

The balance of evidence at the present time would certainly tend to support the view that complete precipitation in chicken precipitin systems does require a NaCl concentration of the order of 8%, and that the effect of such a high ionic strength is to render formed antigen-antibody complexes less soluble and more prone to aggregation and precipitation, the effect being achieved by a salting-out process.

There remains some dubiety concerning the degree of participation of non-antibody nitrogenous compounds in the precipitin reaction, especially since Makinodan et al. (1960) have observed that an apparently normal serum component can and does contribute to the total precipitate. The nature of the antigen-antibody complex certainly requires further elucidation, but at the same time it is not altogether improbable that the comments of Taliaferro (1958) concerning the physico-chemical diversity of immunologically similar antibodies are pertinent to chicken anti-protein systems.

"Antibodies of the same immunological specificity made in the same animal may differ widely in their physico-chemical characteristics, their rate of metabolic decay, their primary union with antigen and their ability to trigger and support the secondary immunological reactions. Such diversity makes it difficult to characterise antibody and impossible to measure total antibody in the serum although excellent relative measures are available."

Added Note : The recent work of Orlans et al. (1961) already referred to, confirms that a high electrolyte concentration is needed for complete precipitation in chicken anti-protein systems. Whereas both 'heavy' and 'light' antibodies precipitated specifically with antigen in 0.9% NaCl, raising the NaCl concentration to 8% resulted in the salting-out of soluble antigen-antibody complexes containing 'light' antibody.

The AbN/AgN of precipitates formed in 1, 4 and 8% NaCl

The AbN/AgN ratios were calculated from the results of analysis of precipitates and are given in Table 9. At each concentration of NaCl the determined N ratios followed a consistent pattern of high value in extreme antibody excess and a lower value in moderate antigen excess. Once the region of considerable antigen excess had been reached the N ratio increased once more.

Although it was at first thought that this peculiar behaviour of the AbN/AgN ratio might be due to experimental errors, the phenomenon was reproduced in later quantitative tests on other pools of chicken anti-BSA serum and it became necessary to consider possible causes for the unusual behaviour of the N ratio curve.

In mammalian systems the normal finding is that the ratio AbN/AgN is high in the antibody excess zone and then falls continuously until it reaches a limiting value in the region of antigen excess. This limiting value is usually regarded as indicating the composition of the antigen-antibody complex having the least amount of antibody that will still precipitate.

In so far as they resemble what has been obtained in mammalian systems the N ratio curves in B and C of Fig. 12 are 'typical'. They depart from normality only in their tendency to rise again in extreme antigen excess. Although curve A looks the most peculiar it may be only quantitatively different in that the rise in the AbN/AgN ratio occurs earlier. It would appear significant that the first three points of all the curves agree quite well. While no adequate reason has yet been found for the apparently anomalous behaviour of the AbN/AgN ratio a number of possible explanations can be suggested.

(i) Any circumstances or conditions promoting or involving non-specific precipitation in extreme antigen excess could account for the increased ratio. The uptake of complement N falls to be considered in this context, but as due steps were taken to circumvent such an occurrence by incorporating versene in the reaction medium it is unlikely that complement N was implicated to any significant extent. It is not altogether improbable that some degree of non-specific precipitation took place in 1% NaCl, for both Hektoen (1918) and Goodman & Ramsay (1957) have drawn attention to the untrustworthiness of precipitin tests carried out in 1% NaCl, due to the occurrence of non-specific reactions.

(ii) The presence in the BSA of a trace component capable of establishing its own immunological system could also have been implicated in bringing about the rise in N ratio. The BSA used for immunisation and for immunochemical analyses was a highly purified preparation (Armour's Crystallised Bovine Plasma Albumin) specified as containing $<0.01\%$ globulins by electrophoresis. The greatest amount of globulin that could therefore have been present in the immunising dose of 47.5 mg BSA would not have exceeded 10 µg protein, a quantity that is extremely minute compared to a conventional immunising dose of several milligrams.

However, Cohn, Wetter and Deutsch (1949), working with conalbumin found that rabbit anti-conalbumin serum invariably contained more antibody directed against highly antigenic impurities than against conalbumin itself. Similar findings were recorded when they investigated the precipitin reaction between egg white and rabbit anti-egg white serum (Wetter, Cohn and Deutsch, 1952). An unexpectedly small proportion of the total antibody in an anti-egg white serum was directed against the four main purified constituent proteins - ovalbumin, conalbumin, lysozyme and ovomucoid -

which comprise more than 90% of egg white protein. Most of the antibody response was to minor poorly defined antigenic components of egg white.

A similar phenomenon has been observed by Augustin (1957). In studying the reaction between immuno-electrophoretically pure BSA and rabbit antiserumshe made use of the tube precipitin test of Oudin (1952). The rabbit antibody was the fixed internal reactant of the system and the BSA was the external reactant. Instead of the anticipated single boundary two distinct advancing edges were obtained, the slower one associated with an even, dense zone of precipitate and the other, moving twice as fast, belonging to a much less dense zone. The sharp onset of the second zone of precipitation was considered good evidence for concluding that the BSA contained a very small amount of impurity to which the rabbit had produced a disportionately large amount of antibody, resulting in a very high Ab/Ag ratio for the impurity and a low Ab/Ag ratio for the BSA itself. Thus the dense and more slowly moving zone represented the BSA system and much less dense and more quickly advancing lower zone was due to the impurity.

In the quantitative precipitin test the influence of a separate antigen-antibody system would not be apparent until sufficient BSA had been added to ensure that adequate quantities of the trace component were available for significant amounts of precipitate to be formed. Thus a region of moderate antigen excess for BSA would represent antibody excess for the contaminant system with a consequent rise in AbN/AgN ratio. Reference to Fig. 12 shows that the reversal of the slope of the N curves did not take place until the systems had achieved considerable excess of BSA, which is in accordance with the expected behaviour of a highly immunogenic trace-contaminant. Further, it would be anticipated that the

TABLE 10.

Analysis of precipitates in a chicken anti-BSA system.

All N values in µg.

Ag N added	Total N pptd.	Ag N pptd.	Ab N pptd.	AbN/AgN
23.3	622	23	599	26
49.5	1010	49	961	20
93	1486	91	1345	15
204	1598	144	1455	10.1
293	1592	135	1457	10.8
395	1421	139	1283	9.2
508	1359	126	1233	9.8
710	1059	87	973	11.2
791	1010	60	950	15.8
887	853	74	779	10.5

The antiserum used in this test was obtained from birds that had undergone immunization with BSA in complete Freund's adjuvant.

behaviour of the N ratio in considerable antigen excess would be exaggerated in the case of antiserum from birds immunised with adjuvanted antigen.

In Fig. 13 and Table 10 are presented the results of a quantitative test in 8% NaCl on a pooled antiserum obtained from a group of young adult birds that had been immunised by 3 or 4 intramuscular injections of BSA in complete Freund's adjuvant (Difco). These results clearly show that the immunisation with adjuvanted antigen did in fact potentiate the rise in AbN/AgN ratio in the antigen excess zone.

(3) The influence of a coincidental precipitin system would be exaggerated by an analytical technique making critical use of trace-labelled antigen, because different serum protein fractions do not label at a uniform rate. Pressman and Sternberger (1950) labelled rabbit antisera with ^{131}I and showed that the albumin fraction labelled three times as fast as the total globulin fraction. The three globulin components also labelled at rates different from one another, in the order $\alpha > \beta > \gamma$.

A similar phenomenon has been noted in trace labelling sera of various domestic animals, including the bovine (Jennings, Lauder, Mulligan and Urquhart, 1954). This would imply a higher specific activity for BSA than for a contaminating globulin, and the application of the BSA-specific activity value to radioactivity measurements on a precipitate containing a significant proportion of the minor component antigen would result in an underestimate of the amount of 'antigen' (BSA + α globulin) present in the precipitate. By subtraction of 'AgN' from total N an overestimate of AbN would be obtained, and the derived AbN/AgN ratio would inevitably be greater than the virtual N ratio. While this postulated explanation is attractive from a theoretical and qualitative

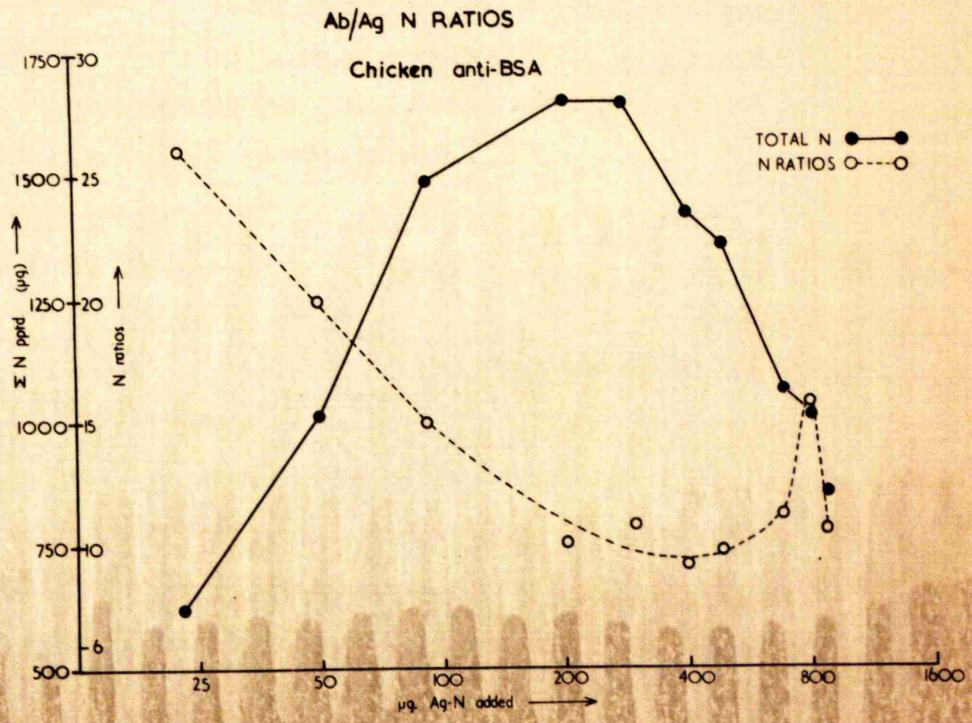

Fig. 13. *The behaviour of the antibody-antigen N ratio in extreme antigen excess is shown in this figure. The serum used for the test was obtained by immunising chickens with BSA in complete Freund's adjuvant.*

view, it fails to stand up to simple quantitative treatment.

(4) The presence of two distinct components in the γ-globulin region of chicken antiserum was noted by Cohn (1952), and the existence of two well-defined antigen-antibody complex peaks was recorded by Banovitz, Singer and Wolfe (1959) when they subjected a solubilised immune precipitate of the chicken anti-BSA system to electrophoresis. This work was confirmed by immunoelectrophoretic experiments of Makinodan, Gengozian and Canning (1960) who found that one of the two complexes contained considerably more antigen than the other. The existence of the two different types of antibody, distinguishable by electrophoresis, is certainly suggested, if not absolutely proven. It is not inconceivable, therefore, that one of these γ-globulin components does not enter into the precipitin reaction until considerable antigen excess has been reached, so contributing to the rise in the AbN/AgN ratio. There is, however, no experimental evidence to support this suggestion. *

An analogous rise in the AbN/AgN ratio was not reported by Wolfe and his colleagues in the chicken anti-BSA systems which they studied, not was there any suggestion from their recorded results that a rise might have occurred. On the other hand the type of analytical figures which they presented did not extend into the region of considerable antigen excess, and therefore precluded the determination of this particular piece of information.

With the anti-BGG system in 8% NaCl there was no indication of unusual behaviour of the AbN/AgN ratio, even though BGG (97% homogeneous on electrophoresis) contains considerably more minor components than does BSA. In this particular case, of course, any anomalies introduced by the relatively faster

* But see 'added note' at end of this discussion.

labelling of albumin than globulin would operate to produce an overestimate of AgN, an underestimate of AbN, and a reduced AbN/AgN ratio. Consequently, any tendency on the part of the ratio to undergo a rise would not be obvious. The effects of injecting chickens with two antigens simultaneously were investigated by Abramoff and Wolfe (1953), using HGG and BSA. The antibody response to HGG was quantitatively normal, while that to BSA was reduced, an effect that was attributed to a 'crowding-out' of the BSA molecule by the larger molecule of HGG. Thus might the antigenic influence of a minor component in BGG have been masked by that of BGG itself. That the minor component or components of BGG possess an effective antigenicity has recently been demonstrated by Dresser (1961). Neonatal mice to which he gave an intraperitoneal injection of 10 mg BGG were rendered simultaneously tolerant to BGG and immune (with antibody production) to a minor component (presumably albumin).

Whatever the nature of the increased N ratio and whatever its cause, there can be little doubt that the phenomenon is a real one. A detailed characterisation must await more extensive analytical work in the antigen excess zone.

Added note: A further alternative explanation of the anomalous behaviour of the AbN/AgN ratios involves the 'heavy' and 'light' antibodies of fowl antisera described by Orlans et al. (1961). These workers found that both antibodies precipitated specifically with antigen in 0.9% NaCl but the increased precipitation at higher salt concentrations was due to the salting out of antibody complexes containing the 'light' antibody. The 'light' antibody was therefore a substance of poor precipitating power in 0.9% NaCl and it was considered that it might be univalent.

The presence of two such antibodies of very different molecular weights and precipitability implies that a change in the AbN/AgN ratio does not necessarily involve a corresponding change in the molecular ratio of antibody to antigen in the complex but could be due to a change in the relative amounts of 'heavy' and 'light' antibody present. The apparent anomaly in the curves of Fig. 12 could be due to this effect.

Considering system A in Fig. 12, in the Ab excess zone both antibodies can be incorporated in the precipitate. However, as the system moves towards the antigen inhibition zone because of its poorer precipitability less of the 'light' antibody will precipitate i.e. the complexes which do precipitate will tend to contain a greater proportion of 'heavy' antibody. A molecule of 'heavy' antibody will contain more than three times the N of a molecule of 'light' antibody so that the AbN/AgN ratio could increase markedly without any increase in the molecular ratio of antibody to antigen. In extreme antigen excess where the complexes precipitated might almost free of 'light' antibody the normal decline in AbN/AgN would be expected to occur. This trend can be seen at the extreme end of the series A curve in Fig. 12.

It is interesting to contemplate how such a situation will vary as the NaCl content of the medium is increased. In 4% and 8% NaCl the 'light' antibody will behave much more efficiently as a precipitant for antigen so that one might expect that the precipitation of considerable amounts of 'light' antibody would continue further into the antigen excess zone than in 1% NaCl, i.e. the anomaly in the AbN/AgN curves would occur much later. Such a conjecture would appear to be borne out by the series B and series C curves of Fig. 12.

Whether the above explanation accounts for all the anomaly in the curves of Fig. 12 or not, it certainly seems

unlikely that the 'light' and 'heavy' antibodies of fowl serum should precipitate in the same relative amounts throughout the full precipitin range, particularly in low NaCl concentrations where their difference in precipitability is most marked. It does not seem likely therefore that chicken precipitin systems will produce AbN/AgN curves which compare closely to those produced by mammalian systems.

Conclusion

Taking into consideration the several aspects of spontaneous precipitation from chicken serum on storage, variation in antibody level with ageing of serum, dissociation of the antigen-antibody complexes with increase in the reaction volume, and the promotion of more complete specific precipitation by higher salt concentrations, there can be no doubt that the behaviour of chicken antiserum differs in several respects from that of its mammalian counterpart.

For immunological studies have been performed in birds and quantitative aspects of avian serology have been studied only in the chicken. What has already been learned is sufficient to indicate the complexity of the subject, but provides adequate stimulus to further investigation. Properly studied, a detailed comparison of mammalian and avian serology holds promise of yielding useful information concerning the in vitro behaviour of precipitating antibody and might throw more light on the fundamental problems of antibody synthesis.

6. SUMMARY OF SECTION

This section deals with serological aspects of the precipitin reaction between the antigens BGG and BSA and their respective chicken antisera.

The incorporation of versene, a chelating agent which removes Ca^{++} and Mg^{++} from solution was found to produce a satisfactory inhibition of coprecipitation of complement N.

Quantitative analyses of the chicken anti-BGG and anti-BSA precipitin systems were carried out using ^{131}I-trace-labelled antigens. The chicken precipitin curves were found to differ in a number of respects from analogous curves for mammalian systems. There was no defined peak of maximal precipitation, a broad plateau being more characteristic in the chicken system. No true equivalence point could be established for in moderate antibody excess precipitation of antigen was incomplete. Throughout the curve the percentage of added antigen actually precipitated varied from almost 100 in extreme antibody excess to 10 or less in extreme antigen excess falling steeply over the region of maximal precipitation. The peculiarities of chicken precipitating antisera raise analytical difficulties which are most easily overcome by the use of labelled reagents.

Determination of the percentage of added antigen precipitated at the point of maximal precipitation, and of the AbN/AgN ratio at this point, formed the basis of a rapid method for the determination of the precipitin content of chicken antiserum, using trace-labelled antigen. The method is of considerable value when large numbers of sera have to be analysed & is particularly useful because only small volumes of serum are required and all analyses are carried out on supernatants. The latter is advantageous when dealing with low titred sera where manipulation, washing and analysis of

a very small precipitate can be difficult.

Quantitative precipitin tests carried out with the BSA system in 1, 4 and 8% NaCl confirmed the observations of other workers on the increased specific precipitation at the higher electrolyte concentrations. A number of tentative explanations for this phenomenon are offered and discussed. An apparent anomaly in the AbN/AgN ratio curves in 1, 4 and 8% NaCl may be explicable in terms of the properties of two physico-chemically different types of antibody in fowl sera.

SECTION D

THE ANTIBODY RESPONSE OF ADULT FOWLS TO CHEMICALLY ALTERED PROTEIN ANTIGENS.

I. INTRODUCTION

Antigenic specificity is known to depend on the chemical structure of the antigen molecule which carries a variable but usually multiple number of reactive sites on its surface. It is to these determinant groupings that antibody activity is directed and it is for them that the antibody molecules develop complementary patterns. The chemical nature of these groups varies from antigen to antigen but it is of particular significance that steric configuration, as well as chemical composition, plays a large role in determining specificity (Boyd, 1956, p. 133). Thus, precipitin reactions can distinguish between two proteins having only small chemical differences, and in fact serological tests can demonstrate the non-identity of proteins found to be similar by ordinary chemical and physical tests. Individual antigens that bear a chemical relationship to one another possess a number of common determinant groupings, the actual number depending upon the closeness of the relationship. For example, functionally similar proteins from related animal species possess similar chemical structures and have many common determinants. Common determinants of this nature are the basis of serological cross reactions (Maurer, 1954).

The introduction of chemical groupings into the antigen molecule can modify antigenic specificity. The technique of chemical alteration of the antigen molecule as a tool for the study of serological specificity was first used by Obermayer and Pick in 1906 (Boyd, 1956, p. 105), but its most extensive application was in the hands of Landsteiner whose thorough and exhaustive investigations established many of the principles of serological specificity (Landsteiner, 1945). In essence, it was found that the addition of simple chemical groups or atoms (e.g. azophenyl, nitro, halogen) to

different proteins would confer a common antigenic specificity. Thus, iodinated ovalbumin when injected into a rabbit would produce antibodies which reacted with other iodinated proteins even though there was no relationship between the carrier proteins themselves. The acquired specificity did not necessarily mask the original specificity of the ovalbumin so that cross reactions could be obtained with the unaltered protein. In their experiments Landsteiner and his associates were able to demonstrate a relationship between the serological specificity of the chemically altered protein and the chemical structure of the molecule introduced. In many cases it was found that the new specificity was not directed entirely to the chemical group added, but involved portions of the protein molecule at the site of attachment of the chemical group, which helped to explain, on the basis of similar spatial configurations, the observed cross-reactions involving proteins treated with different chemical groups.

An important finding in these studies of chemically altered antigens was the discovery that many of the artifically introduced groups, although themselves lacking the ability of stimulating antibody formation, were capable of combining with the specific antibodies induced by immunisation of experimental animals with proteins to which the same chemical groups were attached. Such serological activity indicated that small chemical groups were the basic antigenic units which combined with antibody molecules and were complementary for the specific combining site on the antibody molecule.

The ability of a simple chemical group (hapten) to inhibit the precipitin reaction between a particular chemically altered protein and its antibodies was first noted by Landsteiner, who demonstrated an inhibition reaction in the case of azo-proteins by prior treatment of the antiserum with

a simple azo-dye containing the specific group present in the conjugated protein. The technique of inhibition was also used by Wormall (1930) who applied it to a study of the serological behaviour of halogenated proteins and was able to show that the reaction between iodinated proteins and their antisera could be inhibited by 3:5 diiodotyrosine. It was of significance that 3:5 dibromo- and 3:5 dichlorotyrosine were less active in this respect and that tyrosine itself, several other amino acids, iodophenol and potassium iodide were incapable of inhibiting the precipitin reaction, indicating quite conclusively that the serological specificity of the halogenated protein resided in the 3:5 substituted tyrosine grouping.

These few examples serve to illustrate the dependence of serological specificity upon chemical structure of the antigen and demonstrate the important role played by chemically altered antigens, haptens and the inhibition reaction in solving some of the problems of serological reactions and of serological specificity.

Interest in the potential usefulness of chemically altered antigens to an investigation of immunological unresponsiveness in the chicken was stimulated by certain aspects of the work of Sulzberger and of Chase on experimental drug allergy and of Cinader and Dubert on the problem of specific inhibition of response to purified protein antigens in the rabbit.

Sulzberger's particular interest lay in the sensitising effect of neoarsphenamine (1929, 1930). He found that the intradermal injection of small amounts (150 µg) of this drug would effectively produce a delayed-type hypersensitivity in guinea pigs. He further observed that prior intravenous

injection of the same chemical in larger amounts (6 mg) prevented subsequent sensitisation by intradermal injections. Because neoarsphenamine is unreliable in its sensitising effect no particular significance was attached to his finding at the time, but it is this observation that has relevance to the problem of immunological unresponsiveness.

The later work of Chase was closely related to that of Sulzberger. Certain simple chemical compounds such as picryl chloride and dinitrochlorobenzene, when applied to skin, give rise to an eczematous sensitisation or contact dermititis. Since these chemicals combine readily with protein it is believed that on application to skin they become attached to tissue protein, altering its essential chemical structure and rendering it antigenic to the animal. Such a reaction sets in motion an immunological chain of events which results in the observed dermatitis. Chase (1946) in his studies of this phenomenon found that the oral administration of large amounts of 2:4 dinitrochlorobenzene in oil to adult guinea pigs resulted in a prolonged state of specific unresponsiveness, whereas hypersensitivity to this substance always resulted from intradermal injection in unfed controls. Although it was suggested (Burnet & Fenner, 1949) that passage of the chemical through the gut wall gave rise to a conjugate capable of provoking antibody similar in type to the "protective" antibody produced by parenteral administration of pollen extracts, Chase (1949) considered that this was not the case. He found that such non-sensitive guinea pigs could be passively sensitised by cell transfer, indicating that their refractoriness was not due to the presence of a blocking antibody and that this observation placed the phenomenon more in alignment with the type of unresponsiveness demonstrated by Felton's immunological paralysis of adult mice with

pneumococcal polysaccharide (Felton, 1949).

Sulzberger's original work with neoarsphenamine and Chase's description of the non-reactivity of adult guinea pigs to skin sensitising chemicals induced by prior feeding of the appropriate compound gave rise to the idea that one might be able to produce an analogous situation with a classical antigen-antibody system. The type of experiment envisaged was to see if the immune response to a fully iodinated protein, the determinant group of which is the 3:5-diiodotyrosine (D.I.T.) residue, could be impaired by flooding the animal with simple hapten (D.I.T.) before and during immunisation.

The other experimental findings that were of interest were those reported by Cinader and Dubert (1955). They observed that rabbits injected at birth with human serum albumin (HSA) were, in a number of cases, capable of giving an antibody response to diazo-HSA although unable to form antibody to HSA itself. Further investigations using more sensitive isotopic tracer techniques and antibody detection methods, revealed that this situation arose only in a minority of animals, and that there was some simultaneous production of anti-HSA antibody but that the anti-HSA antibody was of very low avidity (Cinader & Dubert, 1956). However, it seemed possible that, by chemically modifying native protein to varying extents, one might be able to assess the 'degree' of specificity of the tolerance exhibited by an animal made refractory to native protein by embryonic or neonatal injection. If proven sound experimentally, the system might further be applicable to a study of the degree of different types of chemical alteration required to endow a protein with a new antigenic specificity.

Thus the phenomenon described by Sulzberger and Chase, together with the work of Cinader and Dubert, suggested that chemically altered antigens might have a useful role to play in experimental studies on immunological unresponsiveness. In this section of the thesis will be described some preliminary qualitative investigations in the fowl with chemically altered antigens.

2. EXPERIMENT CAA/1.

The aim of this experiment was to determine whether the antibody response to iodinated protein could be interfered with by flooding the animal body with specific hapten (D.I.T.) during immunisation.

Birds

For the experiment 13 birds of the Golden Legbar x Light Sussex variety were obtained and divided into an experimental group of 7 and a control group of 6. These birds were 20 weeks old when the experiment began.

Antigen

Crystalline BSA (Armour & Co.) was prepared in solution and trace-labelled with ^{131}I by the iodine-iodide exchange method. It was thereafter subjected to about 10% iodination as described in the Materials and Methods Section. The solution for injection contained 20 mg protein/ml and was given intravenously. A single dose level of 40 mg was used for all birds. On a body weight basis this was equivalent to c. 30 mg/Kg.

D.I.T.

For iodinated protein the effective hapten is 3:5-diiodotyrosine. Serological studies with rabbit antiserum to iodinated protein have shown that D.I.T. can inhibit the reaction between iodinated protein and its specific antibody (Wormall, 1930). Supplies of D.I.T. (iodogorgoric acid) for administering to birds in the experimental group were obtained from Light & Co. (Colnbrook, Bucks, England). It was given to the birds in two forms.

(i) as an M/10 suspension (43.3 mg/ml) in normal saline, injected intravenously

(ii) in solid form given orally. Powdered D.I.T. was placed in gelatin capsules (size No. 2, Parke-Davis) each capsule containing about 23 mg. D.I.T.

Thus the total daily dose of D.I.T. for each bird in the treated group was 66.3 mg. This dose was well tolerated and no undesirable side effects were observed during or after the period of treatment.

Experimental procedure

Every day for 8 days the birds in the experimental group received a total of 66.3 mg D.I.T. as described. On the second day of this course of treatment both control and experimental groups were injected with antigen. On the 7th, 9th, and 13th days after antigen injection serum was obtained for carrying out qualitative precipitin tests.

Fourteen days after the first injection of antigen a second dose of 40 mg per bird was given intravenously. On the same day treatment with D.I.T. in the experimental group was restarted and went on for 6 days. Serum for precipitin tests was obtained on the 7th, 9th and 14th days after antigen had been given.

A third and final injection of antigen, again 40 mg, was given 14 days following the second one. On this occasion the experimental group received no D.I.T. Serum was obtained on the 3rd and 7th days after injection.

Qualitative precipitin tests were performed as described in the Materials and Methods section. Four solutions of decreasing antigen concentration were prepared by serial x 5 dilution of the most concentrated solution, which contained

TABLE I.

Number of birds giving clearly positive precipitin tests with homologous antigen after 1, 2 and 3 injections of I_2 - BSA.

Group	No. responding after 1st inj.	2nd inj.	3rd inj.
Control	0/6	3/6	6/6
Exptl.	0/7	5/7	5/7

800 µg BSA/ml. All tests on chicken sera were made in a medium containing 8% NaCl.

Experimental Results

After the initial injection of I_2-BSA the sera of all 13 birds, without exception, were found to be negative when tested with I_2-BSA and with BSA (Table 1).

On the 7th day following the second injection of antigen 8 of the 13 birds showed a positive reaction to I_2-BSA; this had waned considerably 48 hours later and had totally disappeared by the 14th day. An almost equal degree of precipitation was observed when the sera were tested with BSA.

There was evidence of an increased production of precipitins in some but not all of the birds on the 7th day after the third injection, and 3 birds produced precipitins for the first time. Cross reaction with BSA was again evident.

Discussion

It had been the intention in this experiment to determine whether the systemic administration of D.I.T. could interfere with the antibody response of the fowl to iodinated protein. On the basis of the results of the qualitative precipitin tests it was clear that both control and experimental birds had produced precipitins to the same degree, and that D.I.T. had apparently had no inhibitory effect.

However, what was particularly striking about the results of this experiment was the complete failure of control birds to produce antibody after a single injection of I_2-BSA and the relatively feeble response even after a second and third antigen injection. Such a poor response was in marked contrast to the prompt and vigorous production of antibody

that characterises immunisation with native protein, and was quite unexpected in view of the fact that iodinated proteins have given good antibody responses in the rabbit in repeated experimental studies. (Banks, Francis, Mulligan & Wormall, 1950; 1951).

It was clear therefore that before any further work with D.I.T. could be carried out it would be necessary to make some investigation of the ability of the domestic fowl to recognise chemically altered proteins as antigens and to respond to them with antibody production. The experiment which followed describes some qualitative work directed along these lines.

3. *EXPERIMENT CAA/2.*

Having obtained only a feeble antibody response from the chicken to repeated injections of iodinated protein it was decided to try to determine whether this incapacity related solely to iodinated protein or whether other chemical modifications had a similar effect. In this study both chickens and rabbits were used. As a species the rabbit is known to be able to produce antibodies specifically directed against the new chemical grouping on a modified protein, so that the immunological response of a few rabbits could be used to make a relative assessment of the response given by chickens to chemically altered antigens. Normally, when pure proteins are used as antigens the fowl is a more rapid and efficient producer of precipitating antibody than is the rabbit.

Birds

When these investigations were started the only birds available were a mixed batch of White Wyandotte and Light Sussex pullets. At the time of the first antigen injection they were 12 weeks old, but at that time and for the duration of the experiment, they showed a greater range of size and body weight than a comparable group of the Golden Legbar cross birds used in most of the work described in this thesis. It is considered that the use of this different variety of fowl was partly responsible for the nature of some of the experimental findings.

Rabbits

Those used in this experiment were adult rabbits of the Dutch variety.

TABLE 2.

Distribution of animals and immunisation schedules for chickens and rabbits used in experiment CAA/2.

Group	No. in Group	Immunising antigen	Route of injection	Days of antigen injection 1st	2nd	3rd
Chickens						
A	19	BSA*	i.v.	1	15	36
B	10	I_2-BSA*	i.v.			
C	10	Br_2-BSA	i.v.			
D	10	PU-BSA	i.v.			
E	12	I_2-BSA(AP)*	i.m.	1	21	40
F	12	BSA(AP)	i.m.			
G	12	I_2-BSA(FA)+	i.m.			
H	12	BSA(FA)	i.m.			
Rabbits						
J	2	I_2-BSA(AP)	i.m.	1	21	-
K	4	BSA(AP)	i.m.			
L	2	I_2-BSA(FA)	i.m.			
M	3	BSA(FA)	i.m.			
N	2	Br_2-BSA(AP)	i.m.	1	17	-
P	2	PU-BSA(AP)	i.m.			

* For the first injection only in groups A and B the antigen was trace-labelled with ^{131}I

\+ (AP) = alum-precipitated

(FA) = incomplete Freund's adjuvant

Experimental procedure

The distribution of animals between groups and the immunisation procedures are given in Table 2. At various time intervals after the administration of antigen blood samples were withdrawn to obtain serum for serological tests.

Each of the birds used received three injections of antigen, the interval between the first and second being either 14 or 20 days and that between the second and third being either 19 or 21 days. The first and second antigen injections were made on a calculated dose level of 50 mg protein /Kg body weight, and for the third injection a dose of 40 mg/Kg was given. At least two blood samples were taken after each injection, one about 7-9 days after injection and the second some 4-5 days later. The sera so obtained were submitted to qualitative precipitin tests and, where positive reactions were obtained with the homologous antigen, the sera were also tested with the other antigens being used in the experiment. In some instances, agar gel diffusion tests were employed to determine the degree of cross-reaction with heterologous antigens.

For the rabbits used in the experiment a similar scheme was adopted. They were given two injections of antigen in adjuvanted form and bled at intervals after each injection. The amount of antigen given at any one injection ranged from 40-100 mg protein.

Experimental Results

1. Chickens: ^{131}I-trace-labelled antigen had been given to 11 of the birds of group A and to all of the birds of group B at the time of the first injection so that a study of antigen elimination patterns could be made. The majority of group A

TABLE 3

Numbers of animals giving positive precipitin responses with homologous antigen after each immunisation.

Group	Immunisation	No.* of animals responding after 1st inj.	2nd inj.	3rd inj.
Chickens				
A	BSA i.v.	1/7	14/17	16/16
B	I_2-BSA i.v.	0/8	2/8	7/8
C	Br_2-BSA i.v.	0/10	8/10	10/10
D	PU-BSA i.v.	0/10	1/10	8/10
E	I_2-BSA(AP)+ i.m.	0/12	10/11	11/11
F	BSA(AP) i.m.	8/11	9/11	8/10
G	I_2-BSA(FA)+ i.m.	1/12	11/11	10/10
H	BSA(FA) i.m.	9/12	10/11	9/11
Rabbits				
J	I_2-BSA(AP) i.m.	2/2	2/2	-
K	BSA(AP) i.m.	3/4	3/4	-
L	I_2-BSA(FA) i.m.	1/2	2/2	-
M	BSA(FA) i.m.	1/3	3/3	-
N	Br_2-BSA(AP) i.m.	2/2	2/2	-
P	PU-BSA(AP) i.m.	2/2	2/2	-

* *The numerator gives the number of animals responding while the denominator indicates the actual number of animals tested.*

\+ *(AP) = alum-precipitated*

(FA) = incomplete Freund's adjuvant

birds (immunised with ^{131}I-BSA) had only a poorly defined immune phase of antigen elimination and significant amounts of activity were retained until 170 hours, which suggested a relatively poor antibody response. The pattern of elimination in group B was markedly different for there was very little activity remaining 24 hours after injection and by 48 hours only background levels could be detected.

Qualitative precipitin tests confirmed that the birds being used in this experiment were not such good precipitin producers as birds of the Golden Legbar cross. Only a minority of birds yielded antibody after a single intravenous injection of BSA (Table 3), but there was virtually a 100% response after a second and third injection, though the amounts of precipitate formed were small in some cases. The adjuvanted forms of BSA were much more successful in eliciting an antibody response to the first injection and subsequent injections served to increase the amount of antibody produced.

Amongst the groups injected with chemically altered antigens it was found that no bird responded to the initial intravenous injection (Table 3). Only Br_2-BSA was able to elicit antibody formation after two injections; I_2-BSA and PU-BSA had to be given for a third time before antibody was detected in the majority of the birds in these groups. A better response to I_2-BSA was given by those birds which received the antigen in adjuvanted form (groups E & G), for practically all of them had circulating antibody after two injections.

When positive antisera were tested with heterologous antigens it was in general observed that antisera to native BSA were always able to cross react with the chemically altered antigens, and that antibodies produced by immunisation with chemically altered antigens were capable of forming

precipitates with native BSA. Antisera to PU-BSA reacted to the same degree with native BSA as with the homologous antigen whereas there was usually a reduced reaction between antisera to halogenated BSA and native BSA as test antigen compared to the reaction between these antisera and their homologous antigens. Br_2-BSA and I_2-BSA appeared to be interchangeable in their ability to precipitate with their respective sera and with anti-BSA, which, in view of their close chemical relationship, was not an unexpected finding.

2. Rabbits: The rabbits used in this experiment gave a better response to the chemically altered antigens than the chickens (Table 3). The majority responded to a first injection of antigen and there was an even better response after a second injection. Serological tests showed that greater amounts of precipitate were found in the homologous BSA system than in systems involving chemically altered antigens.

Antisera to BSA gave noticeably smaller amounts of precipitate with Br_2-BSA and PU-BSA than with native BSA. When I_2-BSA was the test antigen in this system the difference in the amount of precipitate was not so marked. When BSA was reacted with antisera formed by immunisation with chemically altered BSA the amount of precipitate formed was smaller than that obtained with the homologous system.

Discussion

It must be emphasised that the experimental work with chemically altered antigens was qualitative in nature so that only tentative conclusions can be drawn from the results obtained. At face value however, it would seem that whereas the chicken is a very good precipitin producer to native serum proteins this ability does not extend to chemically

altered antigens.

This was first observed in the control group of experiment CAA/I where 3 intravenous injections of I_2-BSA were needed before all 6 birds produced significant amounts of antibody. In the second experiment an attempt was made to determine whether the administration of I_2-BSA in adjuvanted form would promote a more satisfactory response, and whether other methods of chemically altering BSA would result in reduced antigenicity of this protein for the fowl. The results obtained indicated that adjuvanted I_2-BSA injected intramuscularly was more effective in stimulating antibody production than was intravenously injected I_2-BSA. Nor was this latter antigen unique in its failure to elicit a strong antibody response, for similar feeble antibody production resulted from the intravenous injection of Br_2-BSA and PU-BSA, although only two injections of Br_2-BSA were required to initiate precipitin production compared to 3 for the other antigens.

To some extent this experiment was complicated by the fact that the birds being used were relatively poor precipitin producers, even to native BSA, requiring two intravenous injections of BSA before commencing to produce antibody. With the adjuvanted forms of BSA one injection sufficed to initiate antibody production and further injections enhanced the size of the precipitate obtained in serological tests. Fair though the response to BSA was, it failed to equal the rapid and vigorous production of precipitins that is characteristic of the Golden Legbar cross, so that the difference in degree of response to native and chemically altered BSA was not so striking in this experiment. A difference was apparent however, in the number of injections required to stimulate antibody production (cf. Table 3).

For rabbits on the other hand, chemically altered antigens were as effective as native protein in terms of the number of injections needed to start antibody production, though in serological tests the homologous BSA system always yielded more precipitate than systems involving chemically altered antigens.

Contrary to the prolonged retention of ^{131}I-BSA in the circulation of birds of group A there was an extremely rapid clearance of I_2-BSA in group B as determined by antigen elimination studies. The reason for this is not known and further work would need to be done before any definite conclusion could be drawn. The rapid removal of antigen from the blood stream may have some influence on antibody formation.

From the point of view of serological tests, on the chicken antisera it was not easy to tell whether or not the chemically altered proteins had acquired new antigenic specificities. That cross reaction took place between chemically altered antigens and antisera to BSA and also between their antisera and BSA showed that at least part of the immune response was to the carrier protein. There was only slight indication that a new specificity had been acquired in the case of I_2-BSA and Br_2-BSA and none at all in the case of PU -BSA.

With the rabbit antisera less precipitate was formed when native BSA reacted with antisera to chemically altered BSA than in the case of homologous reactions, suggesting that these sera contained antibodies precipitable only with the altered protein i.e. that the chemically modified BSA had acquired a new antigenic specificity.

It bears repeating that these findings are based on qualitative results. Time was not available to carry the work further by studying quantitative aspects of the antibody response to chemically altered BSA, and by making use of inhibition reactions. It had been hoped to use chemically modified protein antigens in investigations of specific immunological unresponsiveness in chickens but the results of these experiments precluded the application of such antigens to the studies already in hand. These two experiments did raise several problems that are worthy of more thorough and intensive investigation. It would seem as though the immune response of the chicken to chemically altered proteins should be studied quantitatively, and also by the use of more sensitive techniques than were employed in the two experiments described. Because only small amounts of antibody may be produced towards the new specific group on the molecule the highly sensitive serological technique of agglutination of tanned sheep red blood cells (Boyden, 1951, Stavitsky, 1954) would perhaps be very suitable for an investigation of this nature.

If the findings of these experiments are corroborated by quantitative studies it is very likely that a comparative study of rabbit and chicken responses to chemically altered and perhaps conjugated proteins will produce some interesting information concerning serological specificity and perhaps about antibody production in the fowl. It is also suggested that the efficacy of adjuvants in the chicken could with advantage be studied for it was remarkable in these experiments that the administration of even native BSA in alum-precipitated form and in oil emulsion did not markedly enhance antibody production.

In view of the interesting information which is coming to light about the properties of chicken antibody (Orlans et al., 1961, and Section C of this thesis) it may be that the physico-chemical conditions necessary for maximum precipitation of antibodies to chemically altered antigens are not the same as those required for complete precipitation of antibody to native serum proteins.

Antibodies to chemically altered antigens may, for example, be more readily precipitated in a conventional electrolyte medium of 0.9% NaCl rather than in 8% NaCl. Suitable investigation is called for to resolve this and other problems of a like nature.

4. SUMMARY AND CONCLUSIONS

Two experiments are described in which chickens were immunised with three different chemically altered forms of BSA. Only feeble antibody responses to these antigens were obtained even after three intravenous injections. A somewhat better response was elicited by one of these antigens administered in adjuvanted form.

Rabbits injected with the same modified forms of BSA were more reliable in their response and appeared to produce more antibody.

Because the findings are based on purely qualitative work it is not possible to draw more than the general conclusion that whereas the chicken is an extremely good producer of precipitins to native serum proteins this immunological ability does not extend to chemically altered proteins.

Further work, of a more detailed and quantitative nature is required in this aspect of the immune responses of the domestic fowl.

SECTION E

STUDIES ON IMMUNOLOGICAL UNRESPONSIVENESS: THE EFFECT OF INJECTION OF ANTIGEN DURING EMBRYONIC AND NEONATAL LIFE.

I. INTRODUCTION

In this section of the thesis will be described several experiments carried out to obtain some precise quantitative information concerning the factors governing the development of immune unresponsiveness in the chicken. Essentially, the experiments involved exposure of experimental animals to antigen at a very early age and a measurement of the immune response produced to the same antigen on subsequent challenge.

At the time when this work was started the available information concerning experimental production of an immunologically unresponsive state in the chicken was limited. In the field of tissue tolerance, Billingham, Brent and Medawar (1953) had unequivocally demonstrated actively acquired tolerance to homografts in adult chickens injected with suitable donor cells during embryonic life. Hasek (1953) had been able to produce a persistent erythrocyte chimerism by the technique of homologous egg to egg parabiosis as also were Billingham, Brent and Medawar, (1956a). Following the disappearance of chimerism there often remained an inability to produce agglutinins to the former partner's red cells (Hasek, 1953). Simonsen (1955) had apparently experienced no difficulty in suppressing the immune response to turkey erythrocytes by injecting chick embryos with turkey blood, though Hasek (1956) was able to achieve this in only one of several birds tested.

Experiments by various workers using microbial antigens in the chicken had produced apparently contradictory findings. Injection of the chick embryo with the viruses of yellow fever (Fox & Laemmert, 1947) and of influenza (Burnet, Stone & Edney, 1950) had had no effect on the ability of the adult bird to produce antibody to the specific virus. At variance with these results are the findings of

Buxton (1954). He inoculated killed Salmonella pullorum intravenously into the developing chick embryo on or before the 15th day of incubation. No demonstrable antibody was formed during the first 80 - 100 days after hatching and there was a marked decrease in the capacity to produce antibody after subsequent oral infection with S. pullorum.

In the opinion of Cohn (1957) any interpretation of these various findings was limited by the essentially qualitative, rather than quantitative, nature of the results obtained. With the aim of determining more precisely whether or not an inhibition of antibody synthesis in the chicken could be achieved by the embryonic injection of foreign antigens, Cohn selected several antigens (4 protein, 1 polysaccharide) whose specific antibodies were susceptible to quantitative measurement. These he injected intravenously on the 14th day of incubation at dose levels of 600 ug or less of protein. In no case was he able to demonstrate a depression of antibody response when the chickens were challenged at the age of 10 - 12 weeks.

The first positive indication that suppression of precipitin formation could be obtained in the fowl came from Hasek's (1956) experiments on hens injected with turkey blood during embryonic life. They remained unable to form anti-turkey serum protein antibody for some weeks and in one case for 6 months. Just as the work described later in this section was beginning there was published a paper by Wolfe, Tempelis, Mueller and Riebel (1957) which quite conclusively demonstrated that a specific unresponsiveness to soluble protein antigen could be brought about in the chicken. By giving very large intraperitoneal injections of BSA to recently hatched chicks these workers rendered the birds incapable of producing an antibody response to BSA for

several weeks, though a complete return of immunological ability was achieved by the 22nd week of life.

Although there were only limited quantitative data concerning the development of immunological unresponsiveness in the chicken, it seemed probable that Cohn's negative results stemmed directly from the use of too small a dose of antigen for the induction of unresponsiveness and too great a delay between the initial exposure to antigen on the 14th day of incubation and subsequent challenge at 10 - 12 weeks. The work of Buxton and of Wolfe et al. indicated quite clearly that depression of immune response in the chicken could be brought about by appropriate antigenic treatment during embryonic or early post-hatching life. A suitable experimental model was therefore available for the investigation of various quantitative aspects of the phenomenon of induced immunological unresponsiveness. The desirability of obtaining quantitative information of this nature has been dealt with in the General Introduction and so too have the reasons for choosing to give the first 'tolerance inducing' dose of antigen during embryonic life. The experiments that were undertaken will now be described and discussed.

2. *EXPERIMENT T/1*

This experiment comprised a preliminary pilot study of the rate and pattern of elimination of a soluble protein antigen from the circulation of normal chickens, and of chickens which had had previous experience of the antigen within a short time of hatching. The purpose of the experiment was two-fold. In the first instance it was desired to learn something of the way in which a foreign protein is handled by the chicken, with particular reference to the application of the antigen-elimination technique in this species as a means of detecting an immune response (Talmage, Dixon, Bukantz and Dammin, 1951). Secondly, an indication was sought concerning the susceptibility of the chicken to the induction of immunological unresponsiveness by neonatal intravenous injection of BGG.

Animals

The chickens used were of the Golden Legbar X Light Sussex cross supplied within 24 hours of hatching. At the time of challenge all birds were 6 weeks old.

Antigen

The antigen, Armour's BGG, was dissolved in sterile normal saline and adjusted to a suitable concentration for injection of newly hatched chicks. Each of two chicks received 1.0 mg BGG in a volume of 0.2 ml, while another pair were given 10 mg BGG in 0.25 ml volume. The antigen used for challenge when the birds were 6 weeks old was trace-labelled with ^{131}I by the iodine-iodide exchange method as described in the Materials and Methods section. Each bird received a single intravenous injection of 31.0 mg ^{131}I - BGG in a volume of 1.0 ml. No correlation between dose size and body weight was made but all dose levels fell in the range 44 - 55 mg/Kg, which is suitable for producing a good

TABLE I

Division of birds between groups and the amounts of antigen injected after hatching and at challenge

Group	No. of birds	Antigen dose in mg	
		Hatching	Challenge
A (i)	2	1.0	31
A (ii)	2	10.0	31
B	6	-	31

precipitin response in normal young adult birds (Brown & Wolfe, 1954).

Experimental procedure

In all, 10 birds were used. These were divided into an experimental group (group A) of 4 birds, while group B, containing 6 birds, were used as controls (Table 1). All 4 birds of group A were given a single intravenous injection of antigen within 24 hours of hatching, using a tuberculin syringe and a 5/8 inch, 28 gauge hypodermic needle. The injections were made into the principal vein of the pelvic limb on the medial aspect of the tarsal joint. At this point the vein is easily seen and because it is reasonably immobile intravenous injection is greatly facilitated. At the age of 6 weeks all 10 birds were challenged with trace-labelled antigen, the injection being made via a wing vein.

Elimination of antigen:

Five minutes after the challenge injection each bird was blood sampled from the opposite wing, by making a small cut with a scalpel blade and allowing about 1.0 ml of blood to run into a small unrimmed glass tube containing powdered heparin. Haemorrhage was controlled by pressure and rapidly arrested once an adequate amount of blood had been obtained. Subsequent samples were taken at 16, 40, 64, 88 and 112 hours after injection, right and left wings being used alternately for venepuncture. By determining the amount of radioactivity in these serial blood samples the rate of disappearance of the labelled antigen from the circulation was studied. The plasma was separated by centrifugation in an MSE refrigerated centrifuge for 30 minutes at 1500 g and at 15°C. Appropriate volumes of plasma were spread on to small nickel planchets as

TABLE 2

Elimination of ^{131}I-BGG by the 6-week old birds in experiment T/I

The figures given represent the amount of radioactivity present in 1.0 ml of plasma expressed as a percentage of the amount contained in 1.0 ml of plasma 5 minutes after injection of the labelled antigen

Group	Bird No	Sampling times: Hours after injection 0	16	40	64	88	112
		Pretreated birds					
A (1)	208	100	32.4	15.8	8.7	-	.001
	209	100	31.1	14.9	9.6	-	.002
A (2)	206	100	31.6	16.5	10.1	-	.003
	207	100	37.3	17.0	10.1	5.3	.006
	Mean	100	33.1	16.1	9.6	5.3	.003
		Control birds					
B	183	100	22.4	15.3	8.2	-	.002
	185	100	33.4	17.6	11.1	3.8	.003
	186	100	34.2	16.4	8.7	-	.002
	197	100	31.0	13.9	7.3	-	.002
	199	100	24.7	13.0	8.2	-	.002
	210	100	29.1	13.8	8.7	-	.001
	Mean	100	29.1	15.0	8.7	3.8	.002

\- not measured

described in Section B. A standard solution was prepared by diluting 1.0 ml of the antigen preparation used for injection to 100 ml with 0.15 M - NaCl to which had been added a small volume of dilute NaOH to keep the protein in solution. From this solution were prepared duplicate standard planchets using 0.1 ml of solution. To eliminate the need for self-absorption corrections in the chicken plasma planchets, normal chicken serum, equal in volume to the amount of test chicken plasma spread, was added to each standard planchet during the spreading of the standard solution. The duplicate standard planchets acted as controls for the natural decay of ^{131}I and enabled all samples to be appropriately corrected. The radioactivity determinations were carried out using a thin end-window G - M counter (EW3H, 20th Century Electronics, Ltd.) After correction for decay all count rates were adjusted to counts per ml of plasma, and the final figures expressed as percentages of the 5 minute sample.

Precipitin response:

On the 8th day after the challenge dose of antigen all birds in the experiment were exsanguinated and serum obtained for determining the nature of the antibody response in the two groups of birds. A qualitative precipitin test was performed, using serial five-fold dilutions of a stock solution of antigen containing 400 µg AgN/ml. The test was carried out in the normal fashion and results read after overnight refrigeration.

Experimental results and Discussion

(i) Antigen elimination: Individual and group mean results for elimination of the challenge dose of antigen are given in Table 2 and group mean results are plotted graphically in Figure 1. From this figure it can be seen that in control birds of group B antigen elimination followed the three-phase

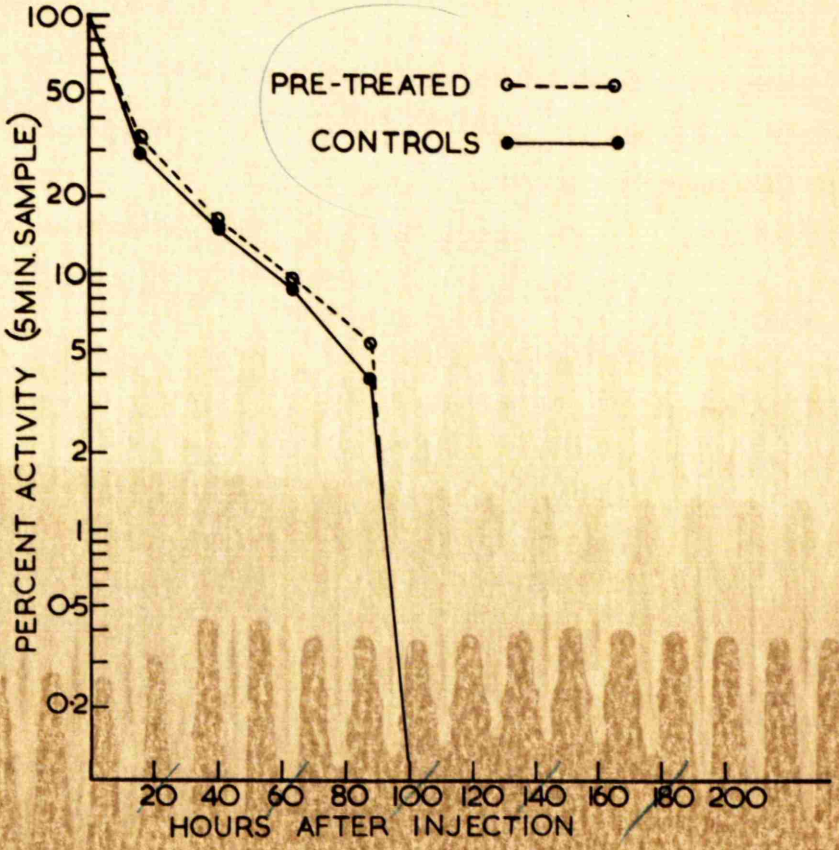

Fig. 1. Elimination of ^{131}I-BGG from the circulation of 6-week old normal birds and of birds that had been injected with antigen on hatching.

pattern described for rabbits by Talmage et al. (1951).

1. Between injection and 16 hours there occurred a "mixing phase" characterised by a fairly rapid drop in circulating antigen due to distribution between body fluids. This phase probably ended before 16 hours but no intermediate samples were taken to establish this point.

2. A constant rate of elimination was observed between 16 and 88 hours, with a half life of 26 hours.* Only one bird was sampled at 88 hours and as this gave evidence of continued catabolic elimination the other birds in the group were not bled at this time.

3. Virtually no antigen could be detected in samples taken at 112 hours, indicating that a more rapid rate of removal of antigen from the circulation had occurred between 88 and 112 hours. Accelerated removal of antigen is characteristic of the "immune phase" that is associated with the release of antibodies into the blood stream.

It is quite clear from these results that the onset of the immune response to BGG is well characterised by an antigen elimination curve, and that this technique can be used to differentiate between an animal producing an immune response and one which fails to do so. In the latter case the phase of accelerated elimination would be lacking and antigen would continue to be removed exponentially from the circulation. Thus an animal unresponsive to a particular protein antigen would retain labelled antigen within the blood stream for a longer period than a normal animal. The retention of circulating antigen by animals only partially tolerant might be expected to fall between these two extremes.

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* While no comperable figure is available for the half-life of homologous gamma globulin in the chicken, Deutsch et al. (1949) found that relatively large amounts of chicken albumin and globulin were rapidly eliminated from the circulation following their intravenous injection.

TABLE 3

Results of qualitative precipitin test on sera taken 8 days after challenge with ^{131}I - BGG

Group	Bird No	Antigen Dilution 1	2	3	4	5	S.C.*
		Pretreated birds					
A (1)	208	++	+++	±	±	-	-
	209	++	++	+	-	-	-
A (2)	206	+++	++	±	±	-	-
	207	++	+	±	-	-	-
		Control birds					
B	183	++	++	±	-	-	-
	185	++	+++	++	±	-	-
	186	+++	++	+	±	-	-
	197	+++	++	±	±	-	-
	199	+	++	±	±	-	-
	210	+++	++	+	-	-	-
Ag control		-	-	-	-	-	

* S.C. = serum control

Individual elimination figures determined for the four pre-treated birds were in close agreement with those of control birds, irrespective of the amount of antigen administered neonatally. The immune drop occurred at the same time and was just as marked as in the control group. It can be concluded therefore, that the intravenous administration of 1.0 or 10.0 mg of BGG to day-old chicks was without effect on the ability of the birds to clear the circulation of a further dose of the same antigen 6 - 7 weeks later, as assessed by the shape of the elimination curve and the rate of removal of labelled antigen.

(ii) Antibody response: Although the pretreated birds manifested an immune clearance of antigen it was not impossible that the primary injection of antigen could have had some significant influence on the immune response either by enhancing or depressing antibody formation. To gain some information on this point all birds in the experiment were exsanguinated on the 8th day after injection of labelled antigen and the sera used for a qualitative precipitin test. The results of this test are given in Table 3. Precipitation occurred in every case and there was no indication of any marked variation in the amounts of precipitate obtained from the sera of pretreated and control birds. Serum control and antigen control tubes were negative.

Although no quantitative determinations of antibody concentration were carried out, the data from antigen elimination curves and from the qualitative precipitin test make it reasonable to assume that the neonatal injection of BGG as a single dose of up to 10 mg within 24 hours of hatching has no effect on the antibody response to challenge with the same antigen 6 weeks later.

3. EXPERIMENT T/2

Although the results of experiment T/1 indicated that a state of durable immunological unresponsive to BGG could not be induced by the intravenous administration of 10 mg protein during the first day of post-hatching life, the number of birds involved was so small as to make the results no more than suggestive. Concerning the question of the size of a successful tolerance-inducing dose, a more condusive answer was sought by a further experiment in which the influence of two aspects of dose effect was studied. The two variables were the magnitude of the total dose given, and the number of injections over which the neonatal dose was spread. The experiment was designed to cover two total dose levels of BGG, 10 mg and 50 mg, each given singly as one injection and cumulatively as five repeated injections at 48 hours intervals.

Also included in this experiment was a small pilot group of 4 birds (group X) that, as 14-day embryos, had received a single intravenous injection (via a chorio-allantoic vein) of 2 mg BGG in a volume of 0.1 ml. Because this was not a composite part of the experiment the data and results relvant to this small group are described and discussed separately (see p.165)

Animals

More than 100 male Golden Legbar X Light Sussex chickens were used in this experiment. They were obtained directly from the hatchery on the day of hatching so that the initial injection of antigen could be given within the first 24 hours of post-hatching life. For the first few days all birds were maintained in the laboratory until the course of neonatal injections had been completed and thereafter they were reared at the Veterinary Field Station by normal methods of poultry husbandry. When challenged, the birds were 9 weeks of age.

TABLE 4

Distribution of experimental groups and schedules of neonatal antigen injections in experiment T/2.

Group	No. of birds	Total Ag dose in mg	Ag injection schedule	Vol & conc^{n} of Ag per injection
A	21	10	5 x 2 mg	0.1 ml of 2%
B	21	10	1 x 10 mg	0.5 ml of 2%
C	21	50	5 x 10 mg	0.5 ml of 2%
D	19	50	1 x 50 mg	0.5 ml of 10%
Control	21	-	-	-

Antigen

Armour's BGG was used in all cases. For the neonatal injections it was dissolved in sterile normal saline to give 2% and 10% solutions. Before use these solutions were centrifuged at 1500 g for 30 minutes and warmed to 37°C prior to injection. Details of neonatal antigen injections are given in Table 4. The antigen used for challenge at 9 weeks was trace-labelled with ^{131}I by the iodine-iodide exchange method, the final concentration for injection being 4.1%. Each bird received 1.0 ml of this solution (41 mg) with no adjustment of the dose level for body weight.

Experimental procedure

Table 4 shows the distribution of groups for this experiment and the schedules for neonatal injections of antigen. The first of these injections was given within 24 hours of hatching and repeat injections in groups A and C were made at 48 hour intervals. Injections were given intravenously using the tarsal vein on the medial aspect of the hock joint. In a very few instances, when carrying out repeat injections in groups A and C it was necessary to resort to the intraperitoneal route when successful intravenous injection could not be achieved. No evidence of systemic disturbance was given by any of the chicks receiving single or multiple injections of antigen, although a few individuals of groups A and C had some bruising and local inflammatory response at the site of repeated injections. This local injury resolved rapidly and appeared not to affect the health and development of the birds.

The elimination of the challenge injection of ^{131}I - BGG from the circulation was studied by determining

the radioactivity of serial blood samples as described in experiment T/1. The first of these blood samples was taken 48 hours after challenge, which represents the middle of the second or 'catabolic' phase of antigen clearance.

On the 7th day after antigen injection all birds were killed and exsanguinated to obtain serum for the quantitative determination of circulating antibody. Serum antibody levels were measured by the method of 'percentage antigen precipitated' discussed in Section C, using 65% precipitation of antigen as the point of maximum precipitation and applying an AbN/AgN ratio of 2.8. With the smallest amount of antigen used in the test, the lower limit of antibody that could be accurately determined was 10µg AbN/ml of serum. Sera which were known from antigen elimination studies to be free of circulating antigen but which failed to give a measureable precipitate with the smallest amount of antigen used in the test, were considered to have less than 10 µg AbN/ml and in calculating group mean antibody levels, a nominal value of 10 µg AbN/ml was assigned to such sera. Where a significant amount (at least 2% of 48 hour level) of circulating antigen was present in the 168 hour sample, it was assumed that the serum was negative in respect of free circulating antibody.

Experimental results

Tables 5 to 9 show the figures obtained in antigen elimination studies and in quantitative precipitin tests. For the sake of clarity of presentation of the data in these tables the animals within each group are arranged according to the nature of the response to the challenge dose of antigen, as determined by study of the rate of its elimination. Those birds showing a well-defined immune

TABLE 5.

Individual and mean results for elimination of antigen and for circulating antibody levels on the 7th day in the control group.

Bird No	Hours after injection						µg AbN per ml serum
	* 48	72	96	120	144	168	
184	100	52	26	5	0.4		28
188	100	47	9	0.3			15
189	100	49	25	2			39
190	100	49	10	0.7			20
192	100	52	10	0.5			38
194	100	58	35	3	0.3		30
196	100	57	23	7.5	0.9		19
204	100	50	15	0.3			37
213	100	36	11	1.1			11
212	100	44	11	3			10
195	100	52	14	10	2		10
198	100	39	25	13	9	4	-
Mean	100	49	18	4			21 ± 13

* 48 hour level of Antigen taken as 100%

TABLE 6.

Individual and mean results for elimination of antigen and for circulating antibody levels on the 7th day in experimental group A (5 x 2 mg)

Bird No.	Hours after injection						µg AbN per ml serum
	* 48	72	96	120	144	168	
101	100	45	11	0,7			29
106	100	41	1	0.2			38
108	100	49	14	0.3			35
110	100	39	3	0.5			30
111	100	51	20	2.2			13
113	100	59	26	0.7			37
114	100	58	17	3.0			34
117	100	48	7	0.4			39
119	100	38	15	0.5			17
120	100	48	10	0.4			32
121	100	31	1	0.0			16
102	100	47	11	1.4			10
105	100	36	7	1.2			10
112	100	54	34	11	0.4		10
115	100	42	14	0.5			10
103	100	54	37	6	3		40
104	100	46	21	11	6	3	-
107	100	50	33	16	16	10	-
109	100	51	31	16	8	3	-
116	100	53	32	19	11	3	-
Mean	100	47	17	4.6			19 ± 14

* 48 hour level of antigen taken as 100%

TABLE 7

Individual and mean results for elimination of antigen and for circulating antibody levels on the 7th day in experimental group B (1 x 10 mg)

Bird No.	Hours after injection						µg AbN per ml serum
	* 48	72	96	120	144	168	
122	100	46	2	2.3			15
124	100	51	14	1.4			16
125	100	48	8	0.4			33
126	100	56	9	0.8			30
127	100	38	3	0.5			34
129	100	55	19	2.0			17
130	100	49	12	0.2			30
131	100	48	12	0.5			23
132	100	51	3	0.1			35
135	100	28	1	0.2			28
136	100	54	20	0.6			12
137	100	49	22	1.4			28
138	100	41	1.0	2.0			31
141	100	50	6.0	1.0			36
142	100	53	10	0.3			28
123	100	56	14	12	0.5		10
128	100	32	10	1.3			10
134	100	43	23	11	8	0.8	10
Mean	100	47	11	2.1			24 ± 10

* 48 hour level of antigen taken as 100%

TABLE 8.

Individual and mean results for elimination of antigen and for circulating antibody levels on the 7th day in experimental group C (5 x 10 mg)

Bird No.	Hours after injection						µg AbN per ml serum
	*48	72	96	120	144	168	
147	100	35	3	0.1			26
152	100	48	17	0.3			31
153	100	59	14	2.0			20
155	100	48	16	6			11
162	100	45	7	0.5			29
163	100	34	10	0.3			28
143	100	49	14	0.5			10
150	100	54	26	8	0.3		10
151	100	43	7	0.4			10
159	100	49	6	0.6			10
145	100	25	13	6	3		10
149	100	41	18	8	4		10
144	100	53	34	19	13	7	-
146	100	52	31	22	14	7	-
148	100	39	19	13	10	5	-
154	100	50	22	12	7	2	-
157	100	55	30	16	10	6	-
161	100	63	26	18	11	4	-
Mean	100	47	17	7.4			11 ± 11

* 48 hour level of antigen taken as 100%

TABLE 9

Individual and mean results for elimination of antigen and for circulating antibody levels in experimental group D (1 x 50 mg) on the 7th day

Bird No.	Hours after injection						µg AbN per ml serum
	*48	72	96	120	144	168	
164	100	41	7	0.7			30
165	100	46	4	0.8			24
166	100	38	2	0			23
167	100	47	14	0.7			14
171	100	55	7	0.6			34
173	100	38	11	2			28
175	100	47	10	0.2			31
177	100	47	22	14	0.3		20
178	100	20	1.0	0.1			34
168	100	53	19	2.3			10
174	100	55	21	4.0	0.6		10
181	100	46	18	6.0	0.3		10
169	100	42	20	8.0	2.0		10
170	100	51	24	12	8.0	4.0	-
172	100	42	25	17	11.0	7.0	-
176	100	44	28	18	-	6.0	-
179	100	49	29	16	10.0	2.0	-
182	100	52	31	20	13.0	8.0	-
Mean	100	45	16	6.8			15 ± 13

* 48 hour level of antigen taken as 100%

TABLE 10

The group mean antibody responses of 9-week old birds 7 days after the injection of a challenge dose of 41.0 mg ^{131}I-BGG

Group & Neonatal Ag dose	No. of birds	µg AbN/ml serum		't' test
		Range	Mean ± S.D.	
Control	12	0 - 39	21 ± 13	
A (5 x 2 mg)	20	0 - 39	19 ± 14	Not sig.
B (1 x 10 mg)	18	10 - 36	24 ± 10	Not sig.
C (5 x 10 mg)	18	0 - 31	11 ± 11	Sig. P<0.5
D (1 x 50 mg)	18	0 - 34	15 ± 13	Not Sig.

elimination phase precede those whose pattern of antigen elimination was not clearly differentiated. Group mean antibody levels determined by the precipitin test are compared statistically in Table 10.

Discussion

(i) Antigen elimination:

In the control group, consisting of 12 birds, virtually complete removal of circulating antigen (< 1% remaining) was achieved by 4 of the birds at 120 hours, and by 6 of the remaining 8 at 144 hours (Table 5). It was clear therefore that the phase of accelerated elimination of antigen that heralds the sudden liberation of circulating antibody did not occur before 100 hours and in the majority of birds was completed by 144 hours after injection of antigen, which correlated with the time of 112 hours determined for similarly treated birds in experiment T/1.

This being the case, an anomaly immediately presented itself, for one of the birds in the control group had a considerable amount of free antigen in the circulation at 168 hours and gave no evidence of an immune drop in the pattern of antigen elimination. Instead it produced the type of antigen elimination curve characteristic of a tolerant animal. The most reasonable explanation appeared to be that the particular individual failed to produce an immune response to the challenge dose of antigen, for similar 'non-responders' to primary injections have occasionally been encountered by Wolfe, (Wolfe & Dilks, 1948; Mueller, Wolfe & McGibbon, 1959), who has found that such birds almost invariably respond normally to a second injection of antigen. A similar refractoriness to a primary course of immunisation with serum protein antigens is seen

TABLE 11

Number of birds in control and experimental groups showing a retention of significant amounts of antigen in the blood stream beyond the time normally required for immune clearance

Group and neonatal Ag dose	No. of birds in group	No. of birds retaining more than 2% * of Ag at	
		144 hours	168 hours
A (5 x 2 mg)	20	5	4 (15) +
B (1 x 10 mg)	18	1	0 (17)
C (5 x 10 mg)	18	8	6 (10)
D (1 x 50 mg)	18	6	5 (12)
Control	12	2	1 (10)

* *Calculated as a percentage of the activity present in the blood at 48 hours.*

\+ *Figures in parentheses are the numbers of birds giving a well defined immune phase of antigen elimination.*

in 10 - 20% of normal rabbits (Dixon & Maurer, 1955). Alternatively it was not impossible that antibody was in fact being produced and that what was circulating was not free antigen but antigen bound to antibody in the form of an antigen-antibody complex which, for some unknown reason, was not being readily removed from the circulation. Because this particular control bird was killed on the 7th day it was not possible to determine whether there was an absolute failure to produce an immune response or whether the production of antibody was merely delayed.

Of the neonatally treated animals only in group B (1 x 10 mg) did all individuals develop a phase of accelerated clearance of circulating antigen. All birds but one had less than 1% of injected antigen remaining at 144 hours, the exception taking 24 hours longer to attain this level. Amongst the three other experimental groups, no one group exhibited complete unresponsiveness in all its members, for in each of groups A, C and D more than half the birds manifested an immune clearance phase. A proportion of the animals in each of these groups maintained the catabolic rate of immune elimination, with a half-life of approximately 24 hours, up to the time of the final measurement at 168 hours. One or two birds in each group seemed to be neither wholly immune nor fully unresponsive, for while no sharp indication of immune clearance was given, yet antigen was more or less completely removed by 168 hours after injection.

On the basis of the rate and pattern of removal of antigen from the circulation the birds in each group could be classified as unresponsive, partially unresponsive, or immune (Table II). If an accelerated phase of antigen

removal took place before 144 hours the birds were considered to be immune, while the retention of significant amounts of circulating antigen until 168 hours, without evidence of an immune clearance phase, was taken as the criterion for unresponsiveness. Those birds whose antigen elimination characteristics fell between these two extremes were classed as partially unresponse. Figure 2 shows typical antigen elimination patterns for the three different types of response.

(ii) Antibody response: Quantitative precipitin tests were carried out on all sera collected at 168 hours. In the case of those known to contain significicant quantities of free antigen it was to be expected that no free antibody would be detected, for failure to develop a phase of accelerated antigen elimination implied that no antibody had been liberated into the circulation to combine with and remove antigen. All animals classed as partially unresponsive on the basis of the antigen elimination study failed to give a definite antibody response. Because the amount of antigen present in the serum at 168 hours was very small, it could not be assumed that no antibody was present, for the smallest amount of antibody capable of accurate measurement was 10 µg AbN/ml of serum. As explained earlier these sera were indicated as having less than 10 µgAbN/ml and although it was probable that some of them possessed no circulating antibody, for the purposes of statistical calculations their antibody level was considered to be 10 µgAbN/ml.

Of the birds that had shown a definite immune elimination phase, a significant number were found not to have measurable quantities of circulating antibody. Why this should have been so was not clear, although it could

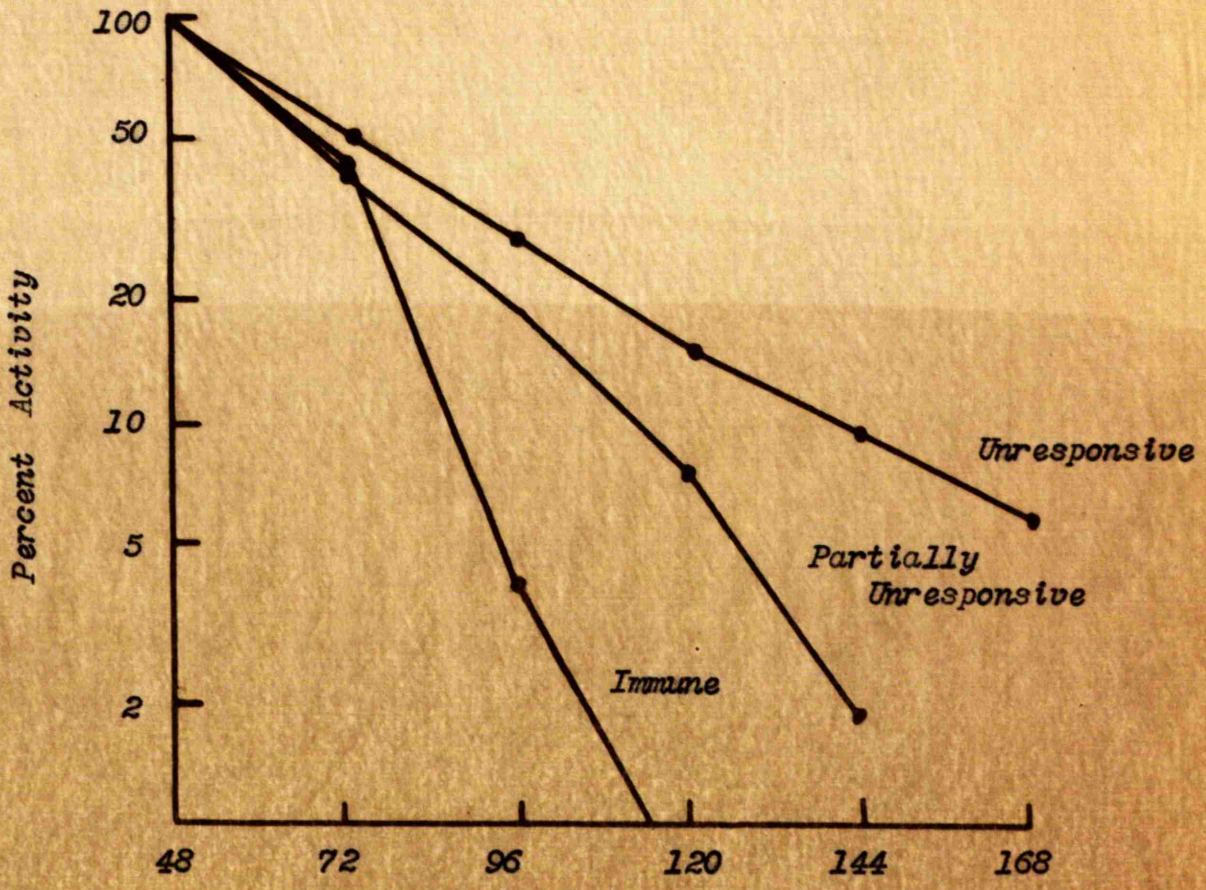

Fig. 2. *Typical patterns of antigen elimination from the circulation of immune, partially unresponsive and unresponsive birds*

have been accounted for by a very short lived antibody response (and chicken precipitin levels do fall rapidly once peak production has ceased, cf. Brown and Wolfe, (1954)) or by the elaboration of an antigen-combining, non-precipitating antibody, though the presence of such antibodies has not yet been shown in the chicken. +

Killing all the birds at 168 hours was in a sense a tactical error because of the limitation imposed on the measurement of antibody production. More satisfactory would have been a procedure of continued serial sampling at 24 - 28 hour intervals to follow the development of serum antibody levels and to determine whether the unresponsiveness indicated by antigen elimination patterns was reflected in a depressed synthesis of antibody or whether antibody production was merely delayed. Such a scheme of serial sampling undertaken in later experiments of a similar nature showed that the antibody response was not, in fact, delayed beyond the usual time.

Given standard conditions relative to the age of the animal at the time of initial exposure to antigen and the interval elapsing before subsequent challenge it seemed reasonable to suppose that there would be a minimum amount of antigen capable of producing a demonstrable degree of unresponsiveness. It was one of the aims of this experiment to determine the order of magnitude of such a threshold dose. For a single neonatal injection, 10 mg BSA was inadequate, and 50 mg was sufficient, to cause a durable unresponsive state in some of the birds given the

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+ The recently published work of Orlans et al. (1961) (see Section C) suggests that fowl antisera do contain quite a considerable proportion of non-precipitating antibodies, which lends support to the second alternative offered above.

respective amount of antigen within 24 hours of hatching. On such evidence alone it would seem that the minimal effective quantity of antigen lay between 10 mg and 50 mg and was probably nearer 50 mg than 10 mg. The difference in efficacy between the two dose levels could be ascribed quite simply to magnitude, the greater dose, ipso facto, exerting the greater inhibiting influence on the antibody forming mechanism. That such an interpretation represents an over-simplification of the case is implicit in the observations concerning the consequences of spreading the same doses over five equal injections at 48 hour intervals, for under those circumstances, 10 mg was almost as competent as 50 mg in the induction of the unresponsive state. The potentiating effect of a divided dose at the 10 mg level provides reasonable evidence that in the induction of tolerance the continued presence of antigen plays a significant role. With a single dose of 10 mg antigen would have disappeared sooner than with 5 doses of 2 mg each at 2 - day intervals. The lack of significant difference between a single and divided 50 mg dose level in tolerance production suggests that the critical factor is not so much persistence of antigen per se as persistence of antigen concentration at a minimum or threshold level. On this basis, a single injection of 10 mg on the first post-hatching day is inadequate to maintain the appropriate level of antigen concentration for a critical period of time. The significance of the results of these experiments is dealt with more fully in the General Discussion of this section.

Group X

As mentioned on page 158this experiment included a small group of 4 birds that had been given 2 mg BGG in 0.1 ml volume on the 14th day of incubation. The technique

TABLE 12

Individual and mean results for elimination of antigen and for circulating antibody levels in 4 birds that had been given 2 mg BGG on the 14th day of incubation and challenged at 9 weeks with 41 mg ^{131}I-BGG.

Bird No.	Hours after injection						µg AbN per ml serum
	* 48	72	96	120	144	168	
201	100	47	18	9	5	2	-
202	100	52	33	20	15	6	-
203	100	60	35	19	15	9	-
205	100	55	31	16	11	1	10
Mean	100	54	29	16	12	5	2.5

* 48 hour level of antigen taken as 100%

of injection is described in the Materials and Methods section. Hatching took place on the same day as the main groups and the four embryonically injected chicks were thus age for age with the rest of the birds in the experiment, and were reared with them. Challenge with ^{131}I-BGG took place at the age of 9 weeks and was followed by blood sampling with exsanguination 168 hours after injection of the antigen, just as for the main groups.

As Table 12 shows three of the birds still had a considerable quantity of circulating antigen at 168 hours. The fourth appeared to develop a phase of accelerated antigen elimination between 144 and 168 hours, although still retaining a detectable quantity of antigen at 168 hours. In no case was free antibody detectable in serum taken 168 hours after antigen injection.

The reason for incorporating this particular pilot group in experiment T/2 was to determine whether embryonic injection of a small amount of BGG could satisfactorily induce unresponsiveness. The results obtained indicated that the method was feasible and apparently had the same order of effect as a neonatal injection of 50 mg of the antigen. This preliminary outcome encouraged further experimentation along the same lines.

4. EXPERIMENT T/3

TABLE 13

Proposed plan of Experiment – T/3

Group	No. of eggs set	Ag treatment given to embryos
A	30	1 mg y.s.* on 1st day
B (i)	50	2 mg i.v.* on 14th day
(ii)	50	2 mg i.v. on 14th day plus antiserum on hatching
C (i)	50	5 mg i.v. on 14th day
(ii)	50	5 mg i.v. on 14th day plus antiserum on hatching
Control	70	nil

* y.s. = yolk sac injection

i.v. = intravenous injection

To learn more about the influence of embryonically injected antigen on the subsequent precipitin forming ability of the immunologically mature bird, a large scale experiment was undertaken. It was planned to inject the antigen at two different times in the incubation period - on the 1st and 14th days - and to vary the amount of antigen given on the 14th day (2 mg and 5 mg). In order to decide whether the presence of embryonically injected antigen circulating in the blood stream (i.e. in an extracellular location) beyond the point of hatching was of critical significance in the degree and duration of any unresponsiveness achieved, it was proposed to administer to some of the newly-hatched chicks sufficient quantities of specific antibody, in the form of antiserum in an attempt to ensure the removal of any remaining antigen. An outline plan of the experiment is given in Table 13.

Animals

A total of 300 hatching eggs of Golden Legbar X Light Sussex strain were obtained from a commercial poultry concern and set in an electric incubator in the laboratory. During the 21 day incubation period routine incubation procedures were adopted. Upon hatching the baby chicks remained in the laboratory for a few days before being transferred to a brooding unit. Subsequent development followed the usual poultry husbandry methods and birds were 81 days old when challenged.

Antigen

Throughout the experiment Armour's BGG was used as the antigen. For all embryonic injections it was prepared in sterile normal saline as a 1% solution and warmed before

TABLE 14

Hatching percentages and baby chick viability in experiment T/3.

Group & Treatment	No. of fertile eggs set	No. hatching	% Hatch	No. of chicks alive at 7 days
A (1 mg y.s.)	30	2	7	2
B (2 mg i.v.)	60	16	27	6
C (5 mg i.v.)	57	7	12	6
Control	60	47	78	45

injection. The preparation used for challenge of experimental and control birds was trace-labelled with ^{131}I as in previous experiments. The final concentration of this solution was 3.6%. Each bird at the time of challenge received 2.0 ml of this solution (72 mg).

Antiserum

Certain groups of newly hatched chicks were given a course of injections of anti-BGG serum which had been prepared in adult birds. The pooled antiserum contained just over 300 µg AbN/ml.

Experimental procedure

(i) Injections of embryos: On the first day of incubation 1 mg BGG in 0.1 ml volume was injected directly into the yolk sac of 30 eggs. This procedure was carried out by drilling a small hole in the shell through which the antigen was injected using an 18 gauge hypodermic needle, the drill hole then being sealed with a drop of paraffin wax. Fourteen-day old embryos were injected via a chorio allantoic vein with 2 mg BGG in 0.2 ml volume and with 5 mg BGG in 0.5 ml volume, using the technique already described in Section B. During this procedure all faultily injected embryos were rejected.

(ii) Incubation and hatching results: The first indication that the experiment could not be carried out as originally planned was obtained when the eggs were 'candled' on the 14th day of incubation. Of the 270 unopened eggs 80 were found to be infertile and were immediately discarded. Consequently the numbers of embryos in each group had to be reduced. Amongst the embryonically injected groups there was an extremely poor hatching percentage as Table 14

TABLE 15

Removal of ^{131}I-BGG from the circulation of 81-day old birds in experiment T/3

Bird No.	Bird Group	Hours after injection of Ag 48	72	96	120
		Experimental birds			
65	A	100	36	1.6	0.5
76	A	100	46.1	7.0	0.7
58	B	100	54.2	10.3	0.4
63	B	100	48.1	9.5	0.4
77	B	100	50.9	6.0	0.6
66	C	100	33.7	1.6	0.4
67	C	100	47.5	4.1	0.4
68	C	100	52.1	23.3	0.6
69	C	100	52.1	15.7	0.5
70	C	100	61.6	25.1	0.8
75	C	100	48.9	16.8	0.5
Mean		100	48.3	11.0	0.5
		Control Birds			
15		100	51.8	4.4	0.4
22		100	53.7	14.6	0.6
25		100	53.3	9.1	0.6
26		100	55.9	21.7	0.8
33		100	44.0	3.5	0.4
37		100	53.1	19.0	0.8
42		100	50.7	7.7	1.0
49		100	53.3	10.1	0.4
56		100	57.3	22.2	1.0
86		100	35.3	1.2	0.3
89		100	56.0	30.4	0.8
Mean		100	51.3	13.1	0.6

shows. Of those that did hatch successfully a number were weakly and died within a few days. By the end of the first week so few experimental chicks were still alive that it was impossible to continue the experiment in its devised form. No alternative remained but to allow the surviving chicks to develop to maturity and then to challenge them. Accordingly at the age of 81 days the 11 surviving experimental birds and an equal number of controls were given a single intravenous injection of ^{131}I - BGG at a standard dose of 72 mg in a volume of 2.0 ml. Elimination of the antigen from the circulation was followed in the usual fashion, the first blood sample being taken 48 hours after injection and subsequent ones at 24 - hour intervals. When all the antigen had been removed from the circulation serum was obtained for performing qualitative precipitin tests. Six antigen concentrations were used in the test and were prepared by progressive five fold dilutions from a stock solution containing 20 mg BGG/ml.

Experimental results

Table 15 contains the data obtained from antigen elimination studies. By 120 hours after injection the amount of residual antigen was very small in every case, and no difference was observed in the group mean antigen elimination patterns.

Serum taken some 18 hours later was used for carrying out qualitative precipitin tests, the results of which are shown in Table 16. There appeared to be no significant degree of difference in the antibody responses of the two groups of birds. No attempt was made to determine quantitative antibody levels.

TABLE 16

Results of qualitative precipitin test performed on sera taken 6 days after challenge of 12-week old birds with 72 mg ^{131}I-BGG

Bird No.	Antigen dilution 1	2	3	4	5	6	Serum control
	Experimental group						
65	++	++	+++	++	+	±	−
76	±	±	+	+	±		
58	−	+	+	+	±	−	−
63	±	+	++	+	±	±	
77	+	+	+	++	±	−	−
66	++	++	+++	+++	++	+	−
67	±	++	++	++	±	−	−
68	−	−	±	±	±	−	−
69	−	±	±	±	±	−	−
70	−	±	+	±	±	−	−
75	−	±	±	±	±	−	−
	Control group						
15	−	±	+	+	±	−	−
22	±	+	+	+	±	−	−
25	±	+	++	++	±	−	−
26	−	±	+	+	±	−	−
33	±	+	+	+	±	−	−
37	−	−	±	±	±	−	−
42	±	±	+	+	±	−	−
49	±	+	++	++	±	−	−
56	±	±	±	+	±	−	−
86	±	+	++	+++	++	−	−
89	−	−	−	−	−	−	−
Ag control	−	−	−	−	−	−	

Discussion

The experiment was seriously marred by the poor hatch resulting from

a) the high percentage of infertile eggs

b) the very considerable mortality amongst injected embryos.

The latter is a common finding in experimental work requiring interference in utero or in ovo, despite the usual precautions which are always taken.

There are two factors which were probably largely responsible for the death of embryos in this experiment. Shock to the embryo at the time of injection is a hazard which cannot be avoided and which is difficult to minimise. Mechanical drilling of the shell, injection of a volume of fluid and cooling of the egg during manipulation are all shock inducing factors. More serious still is the introduction of infection into the sterile egg for invasive micro-organisms are presented with a medium ideally suited to their prolific multiplication. Bacteriological examination of several injected eggs which failed to hatch revealed the presence of diverse organisms. For this experiment the antigen had been prepared under sterile conditions and further precautions had been taken at the time of embryonic injections by using only sterile instruments. Such measures were however, obviously inadequate, and in later work more stringent precautions were adopted.

Because so few birds were involved there are not many conclusions that can be drawn from the results of the challenge injection of antigen. Clearly, no demonstrable unresponsiveness was produced even in birds that had received 5 mg BGG as 14-day old embryos. This was not in accordance with the suggestive information derived from the pilot group of embryonically-treated birds described in experiment T/2, where a marked retention of antigen was observed in birds that had been injected with 2 mg BGG on

the 14th day of incubation. These different findings may have been associated with the time after hatching at which the challenge injection was carried out, the birds in the pilot group of experiment T/2 being 63 days old at this time while those in experiment T/3 were 81 days of age when challenged. It is thus conceivable that any unresponsiveness occurring in the experimental birds of experiment T/3 had waned before the time of challenge.

While this interpretation of the results could be no more than tentative it was clear that further investigation of the effect of embryonically-injected antigen was warranted.

5. EXPERIMENT T/4

In this experiment the induction of immunological unresponsiveness by embryonic injection of antigen was investigated further. Instead of BGG as antigen it was decided to use BSA for it was known that with this antigen a transient phase of reduced antibody response could be brought about by giving large doses to newly hatched chicks (Wolfe et al., 1957). In the rabbit also the majority of successful attempts to establish an unresponsive state were associated with the use of serum albumins, particularly bovine serum albumin (Hanan & Oyama, 1954; Cinader & Dubert, 1955; Smith & Bridges, 1956).

Animals

Fertile eggs of the Golden Legbar X Light Sussex cross were obtained for incubating in the laboratory. The birds hatching from these eggs were reared at the Veterinary Field Station.

Antigen

Armour's BSA was used for injection of 14 - day old embryos, each embryo receiving 5.0 mg BSA in 0.25 ml volume. For the challenge injection ^{131}I - trace-labelled BSA was used, the trace-labelling being performed by the iodine-iodide exchange method. The final concentration of this solution was 3.2% and the dose level of ^{131}I-BSA was 50 mg/Kg body weight.

Antiserum

For the post-hatching injections of immune serum a pooled chicken anti-BSA serum, prepared in adult hens and having an antibody content of over 800 µg AbN/ml, was used.

TABLE 17

Outline plan of experiment T/4

Group	Embryonic injection	Post hatching treatment	No. of birds challenged at 15 weeks
A (i)	5 mg BGG	-	13
(ii)	5 mg BGG	antiserum	7
B (i)	5 mg BGG* + P/S	-	8
(ii)	5 mg BGG + P/S	antiserum	-
C (i)	-	-	20
(ii)	-	antiserum	6

* The antigen solution contained penicillin and streptomycin

Experimental procedure

A general plan of the experiment is given in Table 17. There were three principal groups, A and B consisting of experimental birds and those in group C acting as controls. All birds in groups A and B were given an intravenous injection of 5 mg BGG on the 14th day of incubation.

Stringent precautions were taken in order to avoid a high mortality amongst injected embryos. Following solution in sterile normal saline the antigen solution was Seitz-filtered and distributed in 10 ml lots in antoclaved bottles sealed with vaccine caps. All instruments, syringes and needles were placed in boiling water when not in use, operators scrubbed and disinfected their hands and surgical face masks were worn. Additional to these precautions two antibiotics, penicillin and streptomycin, were incorporated in the antigen solution given to embryos in group B. No antibiotic was added to the antigen solution used for group A embryos as it was not known what influence these antimicrobial agents might exert on the embryo and its maturing immunological system. The possibility of toxic effects could not be ruled out.

It was proposed at the time of hatching to administer antiserum to a number of birds in each group as indicated in Table 17, in an attempt to hasten the removal of residual antigen from the circulation. This was done in groups A and C but because of a poor hatch no birds in group B were available for antiserum treatment, so that B (ii) as shown in Table 17 was dropped from the experiment. Post-hatching injections of anti-BSA serum were carried out in 7 control chicks and in 10 embryonically injected chicks. A total volume of 6.0 ml of antiserum was given to each chick during

TABLE 18

Group mean results for removal of ^{131}I-BSA on challenge at 15 weeks

The figures given refer to amount of activity in the blood as a percentage of the 44 hour level.

Group	No. of birds	Hours after injection of antigen				
		44	68	92	116	140
A (i)	13	100	57.1	31.2	4.4	0.4
(ii)	7	100	56.4	28.9	1.6	0.3
B (i)	8	100	59.9	33.6	3.8	0.4
C (i)	20	100	58.8	28.9	3.1	0.3
(ii)	6	100	59.9	30.6	4.9	0.4
Combined means						
Embryo injected	28	100	57.7	31.3	3.5	0.4
Controls	26	100	58.3	29.3	3.5	0.3

the first 3 days according to the following schedule

1st day	1.0 ml	
2nd day	2.0 ml	
3rd day	3.0 ml	(2 x 1.5 ml with a 6 hour interval)

The antiserum contained, per ml, 250 I.U penicillin and 680 µg streptomycin, and the total dose of 6.0 ml was equivalent to c. 5000 µg AbN.

At the age of 10 weeks all embryonically injected birds were blood sampled and the sera tested for residual antigen with a known potent antiserum, and for antibody to BSA. Negative results were obtained in every case. It had been the original intention to challenge all birds at the age of 10 weeks but for practical reasons this phase of the experiment was forcibly delayed until 5 weeks later. The composition of the groups when the birds were 15 weeks old is shown in Table 17.

Challenge was carried out using ^{131}I-trace-labelled-BSA at a dose level of 50 mg/Kg. Commencing at 44 hours after injection serial blood samples were taken at 24-hour intervals until free antigen was no longer detectable. Radioactivity measurements were carried out using a scintillation counter as described under Materials and Methods (Section B). Serum for the quantitative determination of serum antibody levels by the 'percentage-antigen-precipitated' method was obtained on the 8th day after injection of antigen.

Experimental results

Elimination of antigen was complete in all birds by 140 hours with a well-defined immune phase in every case. The group mean figures for removal of antigen are shown in Table 18.

TABLE 19

Group mean antibody levels in sera taken 8 days after challenge with ^{131}I-BSA (50 mg/Kg).

All figures given refer to μgAbN/ml of serum.

Group	No. of birds	Circulating antibody levels			
		Range	Mean	±	S D
A (i)	13	150 – 952	545	±	263
(ii)	7	405 – 887	587	±	176
B (i)	8	78 – 750	393	±	199
C (i)	20	100 – 715	435	±	145
(ii)	6	242 – 828	487	±	231
Combined means					
Embryo injected	28	78 – 952	517	±	241
Controls	26	100 – 828	424	±	194

Analysis of the sera taken on the 8th day showed that all birds had responded to the challenge injection with precipitin production, and that very good antibody levels had been achieved in almost every case. The group mean antibody levels are given in Table 19. Compared statistically by the 't' test it was found that chicks given antiserum after hatching produced the same amount of antibody as the untreated members of their group, nor was there a significant difference between embryonically injected and control chicks in their response to the challenge injection at 15 weeks.

Discussion

There was a much higher survival rate amongst the embryonically injected birds in this experiment than in the previous one indicating that the greater care taken to avoid the introduction of infection had been of considerable value. The administration of penicillin and streptomycin on the other hand would appear to have exerted a deleterious effect on the embryos, for the hatching percentage in this group was very low, and of the chicks which did hatch a number showed an uncertainty of balance and degree of incoordination that was suggestive of streptomycin toxicity. This could certainly have been possible because, on a weight basis, the amounts of antibiotic used were in excess of therapeutic levels. However the good hatch amongst those embryos which had received antigen alone indicated that with careful precautions additional antibiotic treatment was not necessary.

Because of the unavoidably long period elapsing between embryonic injection of antigen and subsequent challenge with ^{131}I-BSA at 15 weeks it was not altogether

surprising that no evidence of unresponsiveness was obtained at challenge. It was interesting to observe that no significant differences could be shown between experimental and control birds in respect of rate and pattern of antigen removal and of circulating antibody levels indicating that any antibody stimulating effect of the embryo injections was not reflected by a heightened immune response 15 weeks later. Apart from the quite obvious conclusion that 5 mg BSA injected into a 14-day old chick embryo does not result in a state of immunological unresponsiveness that can be detected by challenge at 15 weeks, little else could be gleaned from this experiment and there was clearly a need for further work with this antigen.

6. *EXPERIMENT T/5.*

Having failed to demonstrate an immune unresponsiveness to BSA after giving 5 mg to 14-day old embryos, and having regard to the fact that Wolfe et al. (1957) had clearly shown that in chickens suppression of precipitin formation to BSA could be brought about by post-hatching injections of considerable quantities of antigen, a further investigation of the effect of embryonic injection of BSA was undertaken. The purpose of this experiment was to compare the effect of a very large dose of antigen given to 14-day old embryos with the unresponsiveness developing from serial post-hatching injections of the same antigen, and to assess the duration of the unresponsive state resulting from graded doses of antigen given in the early post-hatching period.

Animals

As in all other experiments the variety of chicken used was the Golden Legbar X Light Sussex. Fertile eggs were set in the laboratory incubator, and when the hatched chicks were 48 hours old they were taken to the Veterinary Field Station for rearing and housing.

Antigen

Throughout the experiment Armour's BSA was the antigen used for embryonic and post-hatching injections and for serological analyses. In one serological test a laboratory preparation of pig gamma globulin (PGG) was employed to demonstrate the specificity of unresponsiveness to BSA.

Antigen solution for injection into embryos and baby chicks was prepared under sterile conditions and warmed to body heat immediately before injection. The concentration of this solution was 8%. For the various challenge injections of antigen in later post-hatching life, BSA was trace-labelled with ^{131}I by the ICI method of McFarlane, as described

TABLE 20

Plan of experiment T/5 showing the antigenic treatment accorded to the experimental groups in the embryonic and post-hatching periods.

Group	Injections of BSA during		
	Embryonic period	Post-hatching period	
	Day 14	Weekly from Day 1	Total dose given
A	-	100 mg i.p.	200 mg
B	-	100 mg i.p.	600 mg
C	-	100 mg i.p.	900 mg
D	40 mg i.v.	-	-
E	-	-	-

in the Materials and Methods section of this thesis.

Experimental procedure

(i) Embryonic and post-hatching injections: The intravenous injection of 14-day old embryos with 40 mg BSA in a volume of 0.5 ml followed the techniques already described. Commencing on the day of hatching and continuing at weekly intervals for different lengths of time baby chicks in groups A, B and C (Table 20) were given an intraperitoneal injection of 100 mg BSA in a volume of 1.25 ml. The total cumulative dose received by birds in group A was 200 mg, in group B was 600 mg, and in group C was 900 mg.

Two randomly chosen embryonically injected chicks were sacrificed 24 hours after hatching, and sufficient blood was collected to give serum for an agar gel diffusion test for the presence of free BSA. Following natural deaths in group D during the first few days after hatching, similar tests were performed with macerated tissues (liver, lungs, kidneys) of the dead chicks.

(ii) Challenge injections: Three separate challenge injections of ^{131}I-BSA were given at 11, 16 and 26 weeks using 40 mg/Kg each time. Representatives of all groups in the experiment were submitted to the 11 week and 16 week challenge, but only members of group C and of the control group were used for the third challenge at 26 weeks. Antigen elimination studies were carried out at each challenge in the manner already described, by taking serial blood samples and determining the radioactivity of these samples in a scintillation counter. At various time intervals after each challenge, more copious blood samples were taken and serum obtained for quantitative precipitin analyses. Circulating antibody levels were determined on the 7th, 12th and 16th days after the first challenge, on the 8th and 11th days

after the second challenge, and on the 10th, 12th and 14th days after the third challenge.

The method used for the quantitative determination of serum antibody was that of 'percentage-antigen-precipitated' using an end point of P_{50} and applying an AbN/AgN ratio of 10 at that point, as described in Section B of this thesis. The activity of the test antigen was such that the smallest quantity of antibody that could be determined with reliable accuracy was 25 μg AbN/ml. For the purpose of statistical analysis any sera producing small but not accurately measurable amounts of precipitate were assumed to have an antibody level of 25 μg AbN/ml if radioactivity measurements of the precipitate showed antigen to be present. Only in cases where radioactivity analysis indicated that no significant precipitation of antigen had occurred was a negative serum antibody level accepted.

Experimental results

(i) Retention of antigen by embryonically injected chicks:

Using agar gel diffusion methods free BSA was readily detectable in the sera of two embryonically injected chicks sacrificed 24 hours after hatching. Positive results were also obtained when macerated tissues of chicks of the same group dying as late as 8 days after hatching were tested in the same way with a potent chicken anti-BSA serum. For all cases the lines of precipitate were confluent with a line formed between the antiserum and a solution of BSA in saline, showing a 'reaction of identity.' These results indicated that BSA, in a form precipitable with specific antiserum was still present in detectable amounts 15 days after injection of 40 mg into the embryo.

(ii) Challenge injections of ^{131}I-BSA at 11 and 16 weeks: The

TABLE 21

Removal of ^{131}I-BSA from the circulation after first and second challenges, the amount of antigen present in the sample being expressed as a percentage of the 18 hour level.

First challenge (11 weeks)

Group	No. of birds	Hours after injection of antigen				
		18	66	90	114	138
A (2 x 100)	8	100	23.2	12.1	4.0	0.3
B (6 x 100(	8	100	23.3	12.5	6.1	2.0
C (9 x 100)	8	100	22.1	11.9	6.9	3.3
D (1 x 40)	10	100	23.4	12.1	4.3	0.9
E (control)	10	100	24.0	11.3	2.5	0.5

Second challenge (16 weeks)

Group	No. of birds	Hours after injection of antigen				
		23	47	71	95	119
A (2 x 100)	8	100	54.3	27.0	5.8	0.5
B (6 x 100)	8	100	55.9	30.3	11.3	3.0
C (9 x 100)	8	100	56.6	34.1	18.4	8.9
D (1 x 40)	10	100	54.0	24.0	4.7	1.0
E (control)	10	100	52.3	25.5	4.2	0.5

TABLE 22.

Details of antibody levels in all groups after first and second challenges with ^{131}I-BSA and results of 't' test for significance of difference between mean antibody levels in control and experimental groups.

Group	No. of birds	µg AbN/ml		't' test	
		range	mean ± SD	P	Sig. wrt controls
7 days after 1st challenge (11 weeks)					
A(2 x 100)	8	50-322	178 ± 101	> 0.4	No
B(6 x 100)	8	0-337	82 ± 115	< 0.05	Yes
C(9 x 100)	8	0-25	19 ± 12	< 0.01	Yes
D(1 x 40)	10	0-450	251 ± 163	> 0.6	No
E(control)	10	101-450	223 ± 123	-	-
8 days after 2nd challenge (16 weeks)					
A(2 x 100)	8	50-724	284 ± 207	> 0.4	No
B(6 x 100)	8	25-200	93 ± 69	< 0.01	Yes
C(9 x 100)	8	0-75	19 ± 26	< 0.01	Yes
D(1 x 40)	10	77-910	496 ± 263	> 0.2	No
E(control)	10	50-657	358 ± 202	-	-

* *Significance with respect to controls*

same birds were used for each of the two challenges. Group mean results for the study of antigen elimination are shown in Table 21 and represented graphically in Figures 3 and 4. From these it can be seen that only groups B and C retained significant amounts of antigen in the blood stream beyond the time of onset of the immune clearance phase in control birds. Further blood sampling of these two groups on both occasions gave evidence of a delayed and poorly defined immune phase of elimination for most of the birds of group B and for a smaller number in group C. Two birds in the latter group failed at both challenges to give any indication of immune elimination and retained detectable amounts of antigen in the blood for about 200 hours after the time of injection.

As mentioned above, serum antibody levels were measured at various time intervals after each challenge with ^{131}I-BSA. It was always the case that the initial sample contained more antibody than later ones, and that later samples showed a progressive decrease in antibody concentration. These findings clearly indicated that in some of the neonatally injected groups the low levels of antibody were not due to a delayed antibody response to challenge injections of antigen. Thus, for the purpose of comparingthe antibody responses following challenge at 11 weeks and challenge at 16 weeks only the measurements relating to the initial sample were used. The results of quantitative determinations of serum antibody levels on the 7th day after first challenge and on the 8th day after second challenge are shown in Table 22. In both cases the mean antibody levels of groups B and C were significantly lower than that of the control group. Group A also had a quantitatively smaller antibody response than the control group but the difference was not of statistical significance, while the mean antibody level of group D was never smaller than that of the control group. The proportions

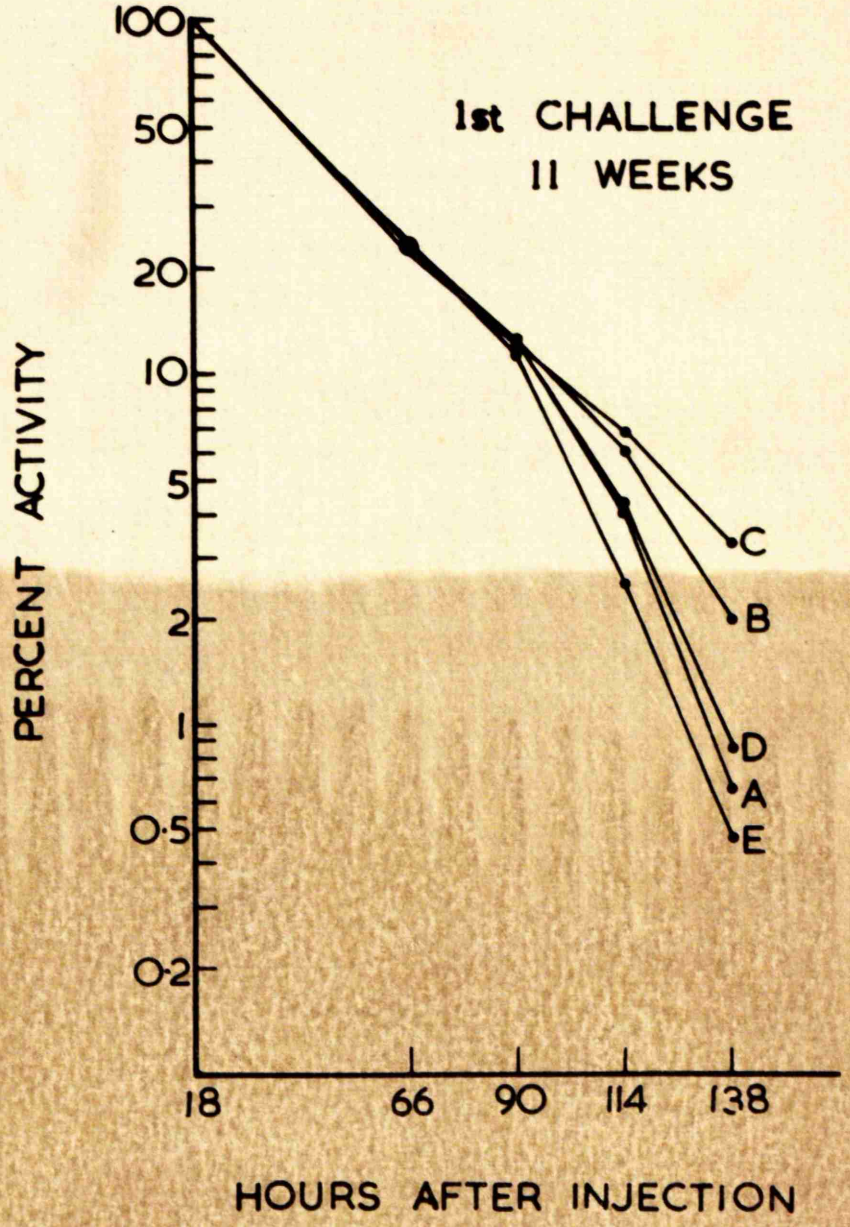


Fig. 3. *Group mean antigen elimination patterns following first challenge with ^{131}I-BSA at 11 weeks.*

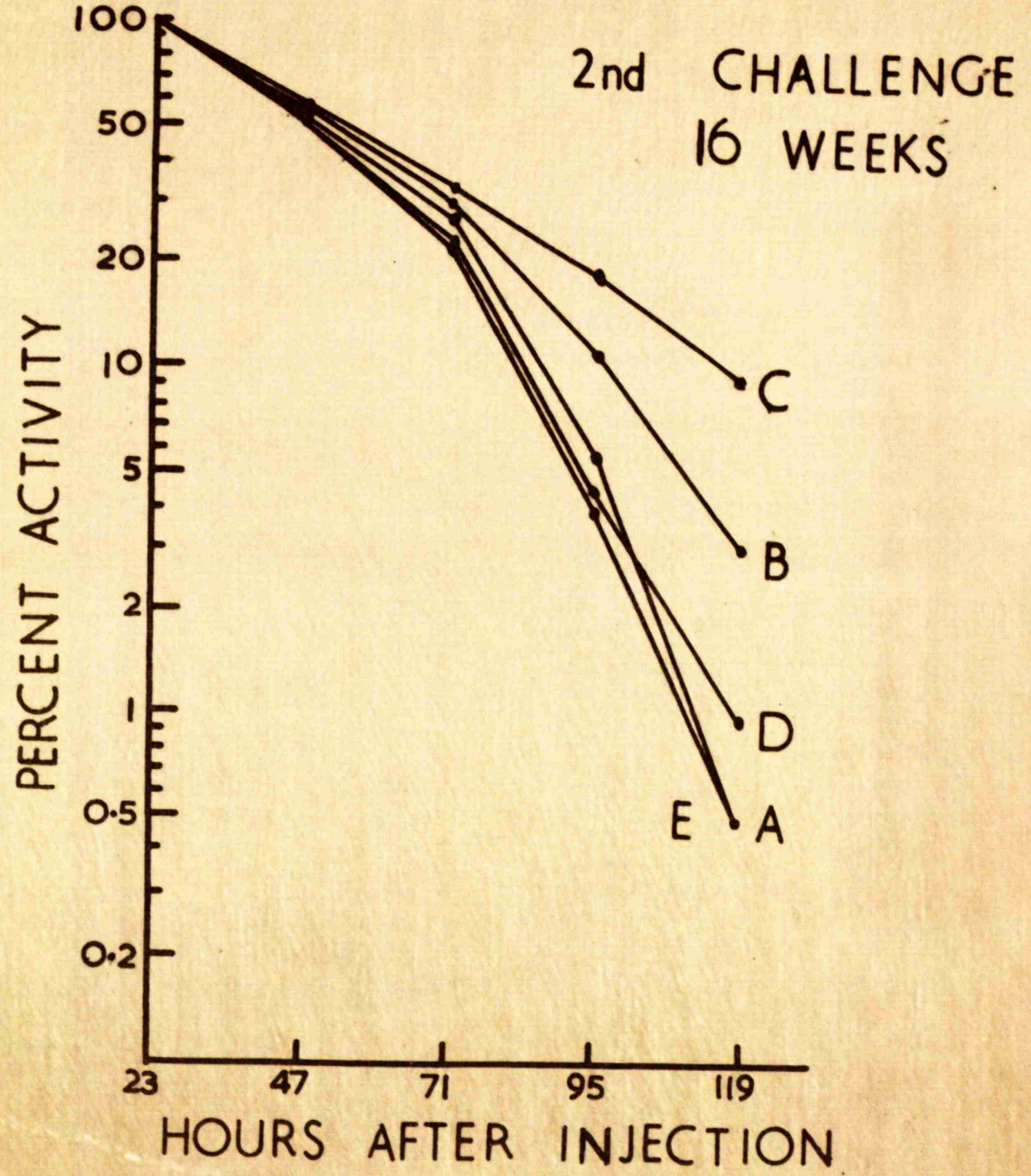


Fig. 4. Group mean antigen elimination patterns following second challenge with ^{131}I-BSA at 16 weeks.

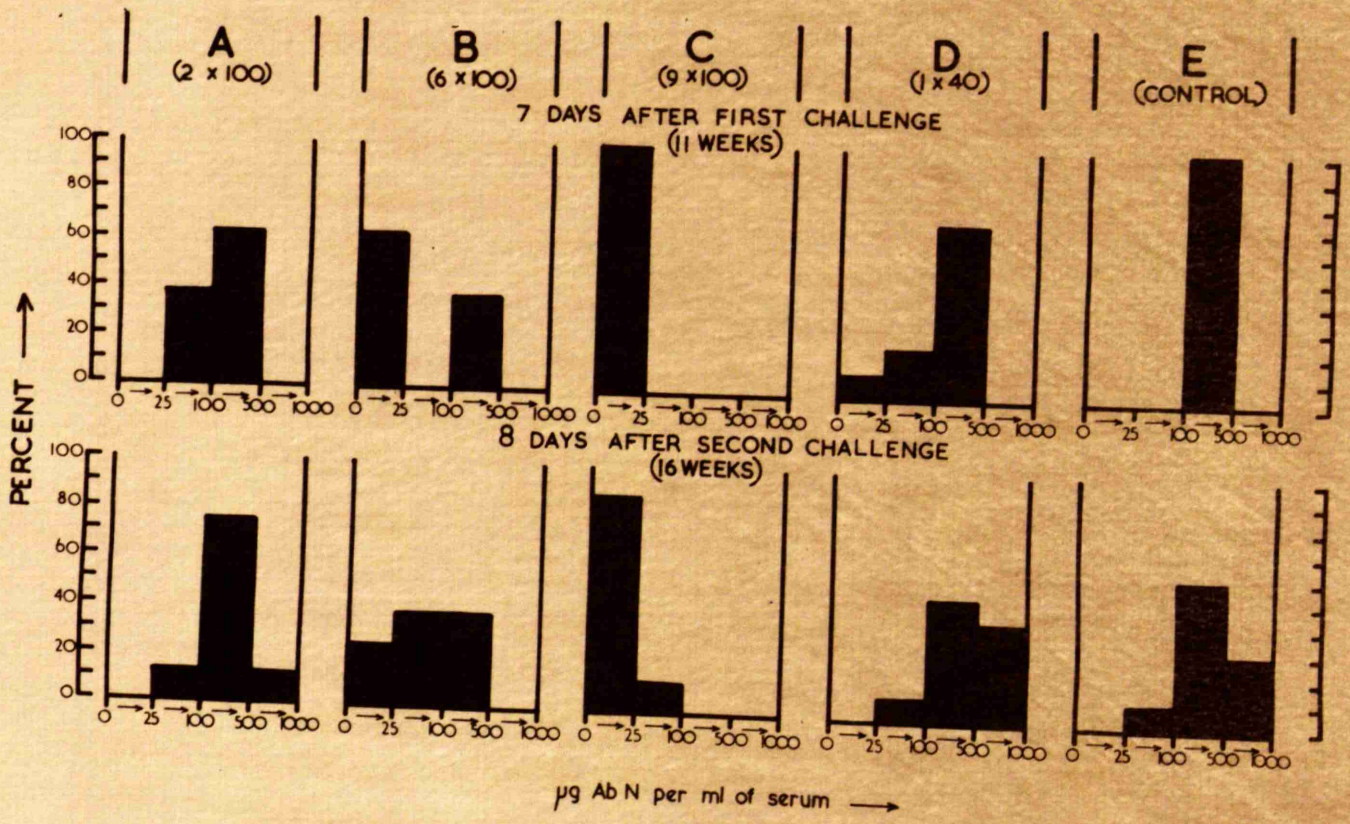


Fig. 5. *Histogram showing the distribution of antibody responses in each group after first and second challenge.*

TABLE 23

Group mean results for elimination of ^{131}I-BSA (^{131}I-BSA (40 mg/Kg) in 26-week old birds, the amount of remaining in the circulation being expressed as a percentage of the 18 hour level.

Group	No. of birds	Hours after injection of antigen						
		18	43	67	91	114	138	162
C	6	100	53.9	30.9	21.8	9.0	4.4	2.2
C_1	6	100	52.4	33.8	25.0	10.3	1.4	0.3
E_1	6	100	51.8	34.9	21.9	6.7	0.9	0.2

of birds giving graded antibody responses after first and second challenges are shown in Figure 5, from which it can be seen that groups A, B and D gave a better response to the second injection of antigen than they did to the first one 5 weeks earlier.

(iii) Challenge injection of ^{131}I-BSA at 26 weeks: Those birds of group C which had already undergone challenge at 11 weeks and at 16 weeks were given a third injection of antigen when they were 26 weeks old. Also challenged at this time were 6 birds from group C that had not received antigen since the end of their series of post-hatching injections (group C_1) and 6 normal untreated birds coming into contact with BSA for the first time in their lives (group E_1). By using these two sets of controls for the third challenge of group C it was hoped to learn

(a) whether the injection of 900 mg BSA in the early post-hatching period could result in a state of unresponsiveness to the antigen that would still be operative when the bird was 26 weeks old, and

(b) whether the amounts of antigen injected at the time of first and second challenges had had any influence on the further duration of the unresponsive state detected in group C birds at 11 weeks.

Group mean antigen elimination figures are given in Table 23 and shown graphically in Figure 6, from which it can be seen that in group C_1 the pattern of removal of antigen from the circulation paralleled that of group E_1, giving evidence of an immune phase of accelerated antigen removal beginning around 114 hours. Though the mean curve for group C failed to show an immune phase an accelerated removal of antigen did occur in 4 of the 6 birds of the group and only 2 continued to eliminate antigen at the catabolic rate

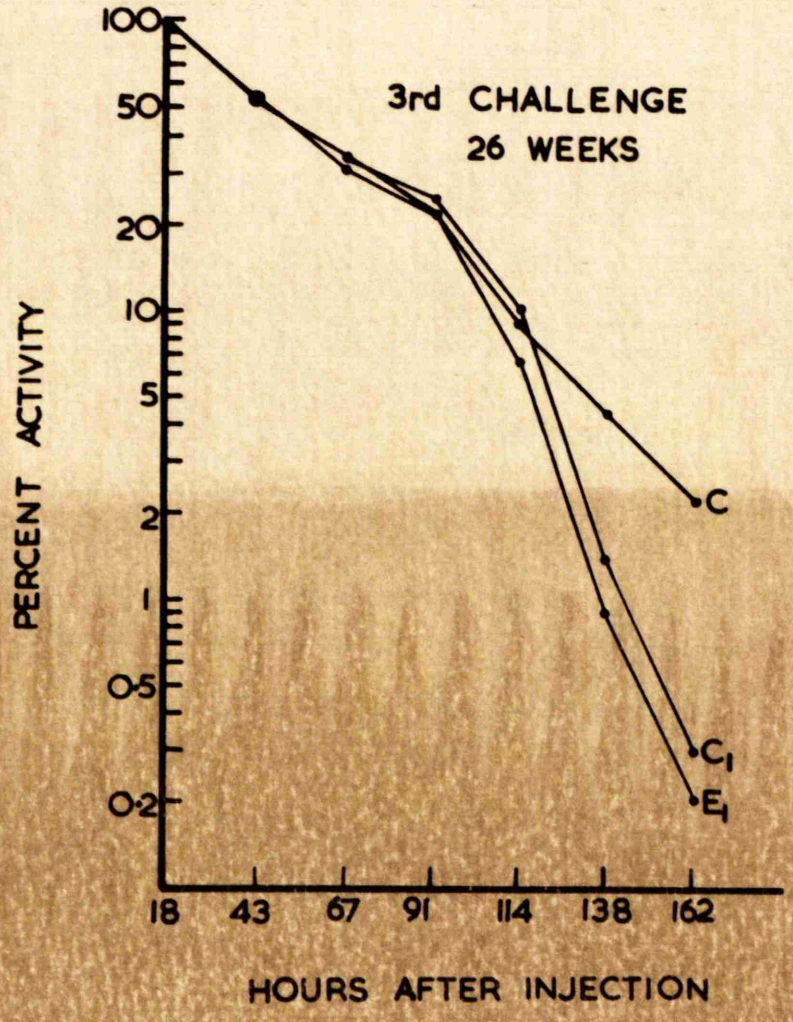


Fig. 6. Group mean antigen elimination patterns following challenge with ^{131}I-BSA at 26 weeks.

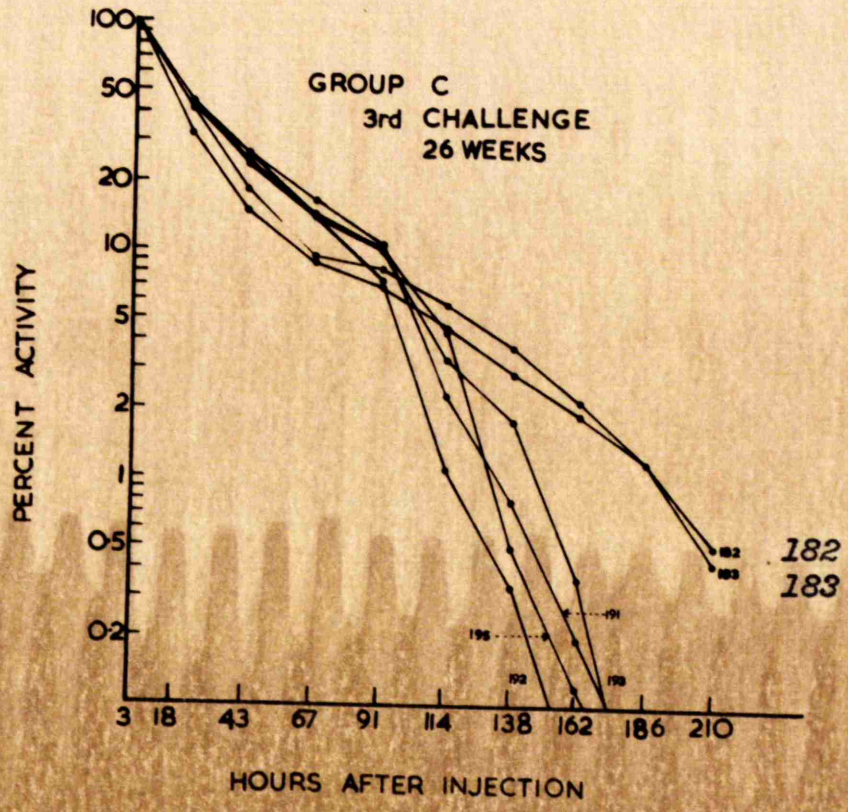


Fig. 7. Individual antigen elimination patterns of birds of group C that were challenged for the third time with ^{131}I-BSA when they were 26 weeks old.

TABLE 24

Individual and mean serum antibody levels in μg AbN/ml on the 10th day after challenge with ^{131}I-BSA at 26 weeks of age.

Group E_1 (normal controls)	Group C_1 (9 x 100 controls)	Group C (9 x 100 3rd challenge)
86	55	0
99	138	0
263	415	170
275	450	315
405	495	401
570	590	415
283	357	217
± 185	± 212	± 189

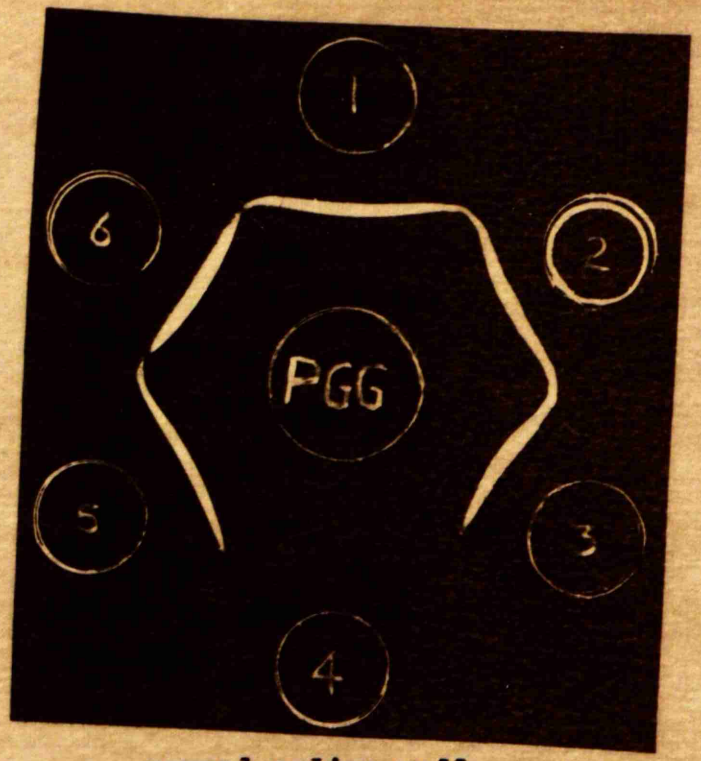
characteristic of unresponsive animals (see Figure 7). Individual and mean serum antibody concentrations are shown in Table 24. A good precipitin response was obtained from the birds in groups E_1 and C_1 and from 4 of those in group C. No precipitating antibody could be detected in the sera of the two birds in the latter group whose antigen elimination patterns had shown no evidence of an immune response. Both these birds (182 and 183) had been completely unresponsive when challenged at 11 and 16 weeks.

To determine whether this state of unresponsiveness would persist without further inducing doses of antigen the two birds were left for a further interval of 12 weeks when it was proposed that their ability to respond to BSA and to another protein antigen should be assessed. Unfortunately one of the two died in the intervening period, the victim of an outbreak of cannabalism, and at the 38th week after hatching only No. 182 remained alive. This bird, together with two other members of its own group and a pair of birds from group E_1, were given immunising doses of BSA and pig gamma globulin (PGG). Serum was obtained on the 8th and 9th days after antigen injection and tested by agar gel diffusion. A typical result of such a diffusion test is shown in Figure 8. Whereas the serum of bird 182 formed a strong precipitate with PGG it did not do so with BSA. The two other members of group C gave sera which precipitated both BSA and PGG, as did the sera of the two birds from group E_1. These finding were taken to mean that bird 182 was still unresponsive to BSA and that the unresponsiveness was specific in nature. It seems probable that bird 183, had it lived, would have given a similar result. No further observations were made and the experiment was concluded at this point.

Bird 182

normal saline well

Bird 182



normal saline well

Fig. 8. *Results of agar gel diffusion test showing on the left the failure of bird 182 to form precipitins to BSA and, on the right, its simultaneous ability to respond to PGG.*

Discussion

A comparison of the effect of a large dose of antigen given to 14 - day old embryos with the specific unresponsiveness following massive post-hatching injections of the same antigen was stated as one of the aims of this experiment. On a group basis it was clear that 40 mg BSA injected intravenously into 14 - day chick embryos was ineffectual in producing an unresponsive state that could be detected by challenge with the same antigen at 11 weeks of age. In this respect embryonically injected birds were similar to those of group A which received 200 mg in the first 8 days of life and which also produced a normal precipitin response at 11 weeks. They differed, however, from those of group B given a total of 600 mg BSA in the first 5 weeks of life for on challenge at 11 weeks the response of this group to homologous antigen was significantly lower than that of controls. Individually, one of the embryo-injected birds, though showing an immune phase of antigen elimination at 11 weeks, apparently produced no precipitating antibody.

That the administration of 40 mg BSA to 14-day old embryos was not without at least a transitory antibody-depressing effect has been strongly suggested by the results of other workers who were also studying the induction of immunological unresponsiveness in the chicken. Stevens, Pietryk and Ciminera (1958) injected 115 mg HSA into the allantoic cavity of fertile eggs after 8 - 18 days of incubation and found the antibody response to challenge at 6 weeks to be significantly depressed. More suggestive still was the work of Tempelis et al. (1958) who found that 37.5 mg BSA injected into 15-day embryos reduced the immune response to the same antigen at 6 weeks, but that 46.8 mg achieved an almost complete inhibition. Very significant

from the point of view of the failure to find a reduced response to BSA in the experiment described in this thesis was the discovery of these workers that at both embryonic dose levels the suppressing effect had completely disappeared by the 12th week. Similar too, was the report of Hirata and Schectman (1960) that 16 - day old chick embryos injected with 50 mg BSA and HGG were unable to respond to these antigens 45 days after hatching but were capable of a normal antibody response at 100 days.

The consistent evidence of these workers makes it almost certain that a degree of antibody suppression would have been observed in group D birds had they been subjected to challenge at the earlier age of 5 - 6 weeks. The same is probably true for group A as Hirata and Schectman found that 50 mg BSA given to 16 - day old embryos and 150 mg injected into newly-hatched chicks resulted in a comparable depression of antibody response to challenge at 6 weeks, and that the effect of both treatments had disappeared by the time the birds were 14 weeks old.

In contrast to these negative results for groups A and D it was found that a post-hatching dose of 600 mg or more of antigen was sufficient to bring about a considerable suppression of the immune response to challenge at 11 and 16 weeks, though by 26 weeks even birds that had received a cumulative total of 900 mg in the first 8 weeks of life were able to give a normal antibody response to BSA. At 11 weeks and again at 16 weeks group B (6 x 100) was found to be less unresponsive than group C (9 x 100) in respect of both rate of antigen removal and production of precipitating antibody (Tables 21 and 22; Figures 6, 7, 8). These findings were taken to mean that the state of immunological unresponsiveness to BSA in chickens brought about by exposing the embryo or

newly hatched chick to large doses of the antigen is a transient, not a permanent, refractoriness, and that the greater the size of the initial antigen treatment the longer does unresponsiveness last.

The results of this experiment were confirmatory of those of Wolfe et al. (1957) who concluded that the administration of large doses of protein antigen to chickens within a short time of hatching is able to suppress the antibody response when the birds are subsequently reinjected, and that the unresponsiveness is not permanent but decreases with age. Further, the duration of the unresponsive period is dependent upon the amount of the antigen administered in early life, since those birds given the greatest dose of antigen remain unresponsive for the longest interval, and the failure of response is specific in nature relating only to the particular antigen given in early life, the ability to produce a normal antibody response to an unrelated antigen being quite unaffected.

Though agreeing in principle the two sets of experimental results presented certain quantitative differences. Wolfe and colleagues were able to demonstrate a significant depression of antibody formation at 12 weeks in birds that had received 100 mg BSA within 30 - 50 hours of hatching, and if the chicks were even younger when they received their initial injection of antigen (112 mg at 8 - 20 hours) a significant reduction of antibody response was still apparent at 22 weeks. In experiment T/5 described here no such effect could be demonstrated even when 100 mg was given to day-old chicks and reinforced by a further 100 mg 7 days later, for at 11 weeks birds treated in this way produced an antibody response that was not statistically different from that of normal control birds of the same age (Table 22). It seems likely that these

differences can be attributed to a differing ability of the breeds of chickens used in the respective experiments to respond immunologically to BSA. Variation between inbred lines of domestic fowl in the production of precipitins by adults is known to occur (Mueller, Wolfe & McGibbon, 1959), and it is reasonable to assume that similar variations might account for what are quantitative, nor qualitative, differences in the experimental results of Wolfe et al. and those recorded here.

The results of the third challenge made it apparent that the unresponsiveness to BSA in group C (9 x 100 mg), clearly demonstrable at 11 and 16 weeks, did not last as long as 26 weeks. All 6 birds which were challenged for the first time at 26 weeks responded with an antibody production equivalent to that of normal control birds, as did 4 of the 6 birds receiving their third challenge at this time, indicating that even when the post-hatching course of antigen injections was reinforced by 40 mg/Kg at 11 and 16 weeks the unresponsive state had waned in the majority of birds. On the other hand the fact that 2 of the 6 birds undergoing challenge for the 3rd time at 26 weeks failed to give an immune response suggested that in at least a proportion of birds the challenge doses were adequate to maintain the unresponsive state. Even 12 weeks later the one survivor of this pair of birds was unable to produce precipitins to BSA though simultaneously capable of a normal response to PGG.

That the third challenge dose should be capable of maintaining unresponsiveness for such a comparatively long time was rather surprising, for in most of the birds given similar antigenic treatment in early life and at challenge, the effect of an equivalent amount of antigen injected at 16 weeks had completely disappeared 10 weeks later. However,

it is noteworthy that both of the birds remaining unresponsive at 16 weeks (Nos. 182 and 183) had failed absolutely at all times to produce antibody whereas even at 11 weeks a feeble response was given by other members of the group. It would appear that only in these two birds was a fully unresponsive state achieved by the serial post-hatching injections and that the immune response of the other members of the group was incompletely suppressed so that they were only partially unresponsive and therefore capable of a more rapid recovery as evidenced by their normal antibody response at 26 weeks. While it is not known for certain whether the periodical challenge injections actually prolonged the unresponsive state in birds 182 and 183 the fact that not one of the birds challenged for the first time at 26 weeks was unresponsive suggests that such was probably the case.

This interpretation of the experimental observations suggests that once a complete unresponsiveness has been induced it can be relatively easily maintained by small reinforcing injections of the antigen, whereas if unresponsiveness is incomplete it is necessary to administer very much larger doses of antigen in order to subdue the immune response for an equivalent length of time. In other words the difference between absolute and partial unresponsiveness is quantitative, not qualitative. It is further implied that between the amount of antigen needed for the inception of the absolutely unresponsive state and that required for its maintenance there is a vital and significant quantitative difference, an implication that is in accordance with contemporary developments in studies of the phenomenon of tolerance in other immunological systems (Medawar, 1961).

It is acknowledged that the evidence for this

interpretation rests on results obtained with relatively few birds, butthe findings are considered to be of sufficient significence to justify further investigations of a similar nature using the domestic fowl as an experimental animal. A more precise resolution of the difference between complete and partial tolerance, and between the quantities of antigen required for inception and maintenance of the unresponsive state have become fundamental to a more complete understanding of this particular aspect of immunology. Most suitable for appropriate experimental investigations of these differences is the simple immunological system involving a defined protein antigen, and an experimental animal such as the fowl in which unresponsiveness is relatively short-lived, so that long-term experiments are not called for.

7. *EXPERIMENT T/6*

AN ATTEMPT TO MAKE PIGS SPECIFICALLY UNRESPONSIVE TO BOVINE GAMMA GLOBULIN

The different degrees of maturity of various species at the time of birth make it clear that there must be important species differences in the ease with which the unresponsive state can be induced in newly born animals. This has been shown to be the case for 'actively acquired tolerance of homografts'. For example, rats can be rendered tolerant by the intravenous injection of living tissue cells for as long as fourteen days after birth, for mice the equivalent period ends shortly after birth, while in sheep, foetal lambs of 100 days or more can successfully reject homografts.

Work with soluble protein antigens has not extended beyond the common laboratory animals, though it is desirable that the phenomenon of unresponsiveness be investigated in as many species as possible. During the course of studies on the immunological responses of young animals two litters of pigs became available for experimental work, and opportunity was taken to determine what might be the influence of neonatally injected antigen in this species. Modelling the experimental plan on the protocol adopted for chicken experiments, a foreign protein was administered to newly born pigs which, after achieving relative maturity, were exposed to challenge with the same antigen.

Animals

In this experiment were used the offspring of two Large White sows that farrowed within nine days of each other with litters of twelve and thirteen respectively. A total of thirteen piglets, six from one litter and seven from the other, formed the experimental group, and the

remaining six in each litter constituted a control group of twelve animals. In accordance with standard pig husbandry procedure the litters remained with their dams until weaned at eight weeks of age, when they were transferred to the fattening house. In the course of this time, six animals died. Within the first few days of life four were crushed to death by the sows and in the post-weaning period another two were destroyed because of unthriftiness and failure to maintain profitable weight gains. At the time of challenge, the elder litter was 144 days old, the younger 135 days.

Antigen

The foreign protein used as antigen was BGG. For the injection of newly born pigs Armour's BGG was prepared in sterile normal saline as a 10% solution. For the challenge injections, BGG was prepared in the laboratory from pooled bovine serum, Seitz filtered, adjusted to a concentration of 2% with sterile normal saline, and distributed in sterile sealed vaccine bottles. Until required, all bottles were held at - 20°C. Once a sealed bottle was opened it was not returned to the original batch whether the contents had been used or not.

Experimental procedure

Each newborn piglet in the experimental group was given a single intravenous injection via an ear vein of 500mg BGG in a volume of 5.0ml. In two cases a volume of up to 2ml was inadvertently administered subcutaneously instead of intravenously. These small volumes of subcutaneously deposited antigen were absorbed within 24 hours and subsequent results showed that the two piglets

so injected did not differ immunologically from the remainder of the experimental group. The neonatal injections were made very soon after birth at 1½ hours and 5 hours respectively after the sows had farrowed. The challenge dose consisted of 4 injections, each of 200mg (10ml of a 2% solution), with an interval of 5 days between each injection so that each pig received a total dose of 800mg BGG. All injections of antigen were by the intraperitoneal route. Because of the risk of introducing infection into the peritoneal cavity by the process of antigen injection, it was considered necessary to carry out prophylactic antibiotic treatment. Accordingly, at the time of the primary challenge injection, each pig received 150,000 IU* crystalline penicillin (Crystapen-Glaxo) by the intraperitoneal route, together with 300,000 IU of 'retarded absorption' penicillin (Mylipen - Glaxo) intramuscularly. On each of the three subsequent days when antigen was administered, 450,000 IU of mylipen only was given.

Ten days after the fourth and last dose of antigen all the pigs were bled from the ear. Only small quantities of blood were available and it was not possible to repeat the sampling. Sera were removed and kept for the determination of antibody levels by a qualitative precipitin test. This test was performed in the usual manner, with the 4 antigen concentrations listed below:

Antigen No.	mg protein/ml
1	40
2	20
3	4
4	0.8

*IU = international units.

TABLE 25

Results of the qualitative precipitin test on pigsera taken 10 days after the last challenge injection.

Pig No.	Antigen 1	2	3	4	Serum Control
Experimental group					
1	+	++	-	-	-
2	++	+	+	-	-
4	+	++	+	-	-
5	+	++	+	-	-
6	++	+	+	-	-
13	+	+	++	-	-
14	+	+	+	-	-
15	+/-	+	-	-	-
16	+/-	+	++	+	-
17	+	++	+	-	-
Control group					
7	+	+	++	-	-
9	+/-	+	+	-	-
10	-	+	-	-	-
11	+	+	+	+	-
12	++	++	+	-	-
20	+	++	+	-	-
21	+	++	+	-	-
22	+/-	++	+	+	-
24	+	++	+	-	-
Ag control					

Antigen solutions were reacted with constant volumes of serum, incubated at 37°C for 2 hours and kept overnight at 5°C before the test was read.

Experimental results

Table 25 illustrates the results that were obtained with the qualitative precipitin test. Precipitation occurred with every serum but was not marked in any one case, the response being best described as moderate. There was no apparent difference in the responses given by the two groups.

Discussion

Only a single blood sample was taken after challenge and only a qualitative precipitin test was performed on this sample. Though quantitative information was obviously more desirable the absence of any suggestion of unresponsiveness in the pretreated pigs was taken to indicate that any quantitative differences between the two groups would be of no significance. Further analysis was not therefore undertaken.

On the results available it was evident that the administration of 500mg BGG to baby pigs within 6 hours of birth did not prevent them from producing an immune response to the same antigen when challenged at the age of 20 weeks. Whereas one cannot assess the nature of an immune response on one qualitative precipitin test it was observed that sera of control litter-mates did not differ materially in the amount of precipitate formed with the test antigen when compared to the sera of pretreated pigs. It was thus assumed that the pretreated piglets had produced a primary immune response on challenge, and that whatever might have been the effect of

neonatally injected antigen it had not at that time resulted in antibody formation of any significant degree. Whether a period of immune unresponsiveness to BGG followed its administration shortly after birth was not known, but it was clear that any such period had fully terminated by the time the pigs were 20 weeks old.

Despite the widespread use of vaccines and antisera in veterinary practice there is very little factual information about the basic immunological behaviour of domestic animals. Clearly there is a need for appropriate fundamental studies with simple antigens in the various species both in respect of normal immune responses and of unresponsive states. For this latter type of work, the pig is quite suitable in view of the large litter size and because it is relatively simple to carry out intravenous injections in newborn piglets. Although no evidence of unresponsiveness was obtained in the case described here further work with other antigens is indicated.

8. GENERAL DISCUSSION

General Discussion

The state of tolerance, once properly established, can last a life time as the study of naturally occurring red cell chimerism has shown (Owen, 1945). Similarly the longevity of skin homografts in experimental animals rendered tolerant by the injection of foreign tissue cells during foetal or neonatal life is attributable to the development of a chimerism (Billingham & Brent, 1957). Survival of the injected cells into adult life means that the antibody producing mechanism of the host is faced with a "chronic antigenic stimulus." Whether persistence of antigen is a prerequisite of the tolerant state is another matter that can be properly investigated only by using inert non self-replicating antigens. For this and other problems of immunological tolerance the system most amenable to quantitative investigation is the precipitin system, using a defined protein as antigen.

In the experiments described in this section of the thesis such an immunological system was used in the domestic fowl to study certain aspects of the state of specific unresponsiveness in this species. One of the most striking pieces of information arising from these studies is the relatively great difficulty experienced in inducing a specific immunological unresponsiveness to a protein antigen in the fowl, compared to the rabbit, mouse and guinea pig. In rabbits for example, BSA in amounts exceeding 10 mg given at birth will inhibit the immune response for some 90 - 120 days (Smith & Bridges, 1958) whereas even 20 times that amount is without effect in the chicken. On the other hand the resistance of the fowl to the induction of an acquired immunological unresponsiveness is not altogether surprising in view of its particular ability to produce precipitins to this antigen in adult life, and it does have the merit that

unresponsiveness, once induced, is of comparatively short duration. This, coupled with an early development of a precipitin producing capacity which gradually increases until the bird is 6 months old, makes the fowl a very useful experimental animal for studying quantitative aspects of tolerance with soluble protein antigens.

In the earlier work described in this section, experiments T/1, T/2 and T/3, BGG was used in attempts to establish a base line for the induction of unresponsiveness by injecting the protein into newly hatched chicks. Unresponsiveness was assessed by studying the removal of a challenge dose ^{131}I-labelled antigen from the circulation and by measuring the precipitin response. By these methods 10 mg given as a single dose was found to be ineffectual but 50 mg was able to induce at least a partial unresponsiveness in a significant proportion of birds. A better result was achieved by giving the 10 mg not as one dose but as a number of small doses over five 2-day intervals. In this way a greater number of birds were made unresponsive, the cumulative 10 mg dose being almost as efficient as the 50 mg one. Dividing the 50 mg dose in a similar way did not improve its ability to induce unresponsiveness. Thus it was established that for a single dose the minimum or threshold amount of BGG that has to be administered to baby chicks in order that a signficant proportion of them shall be incapable of a normal immune response to challenge at 9 weeks lies somewhere between 10 and 50 mg. The enhancing effect of dividing the dose over several injections, especially noticeable at the 10 mg level, is an interesting one that calls for its own interpretation.

Though limited factual evidence is available it is generally accepted that the effect of neonatally injected antigen is experienced at the cellulular level, the antigen or

a derivative penetrating the cell and in some way inhibiting the process of antibody formation. In due course the inhibitory effect is terminated, probably as the result of depletion of available antigen reserve, and antibody formation ensues. The actual mechanisms involved, though the subject of much speculation, remain unknown. It is, however, well established that it is easier to induce unresponsiveness in young animals than in ones which have reached immunological maturity. Why this should be so is uncertain though it may be concerned with the relative susceptibilities of immature and mature antibody forming cells to excessive amounts of antigen for it is during this early phase of the animal's life that, according to Burnet's hypothesis (Burnet, 1959), the immunological mechanism is preoccupied with learning to recognise 'self' and is not capable of a normal response to 'foreign' material. On the other hand the difficulty experienced in making adult animals unresponsive may be merely a quantitative effect related to a greater numerical availability of antibody forming cells in the adult animal. It is also known that during the first two weeks of life the immunological system of the chicken undergoes considerable development as shown by the acquisition of the ability to reject skin homografts (Canon & Longmire, 1952) and by the increasing ability to produce precipitins (Wolfe & Dilks, 1948).

To induce unresponsiveness in the chicken the results of the experiments with BGG mentioned above suggest that it is important to maintain a critical level of antigen in the animal during the early post-hatching period. This level can be attained by giving 50 mg within 24 hours of hatching or by injecting a cumulative dose of 10 mg in 2 mg doses at 48 hour intervals, starting at the time of hatching. By maintaining a suitable level of antigen in the blood stream during first

few days a greater number of developing antibody-forming cells are exposed to a critical concentration of antigen than would be the case with a single small injection (10 mg) given on the day of hatching. For the same reason a single intravenous injection of the embryo would be expected to have no significant tolerance inducing effect and this was an almost consistent finding in the experimental studies described in this thesis. The only evidence of unresponsiveness following embryonic injection was obtained in a small pilot experiment with BGG and in that case the numbers were too few to be of real significance.

Both Wolfe et al (1957) in chickens and Dresser (1961) in mice compared the effect of multiple doses versus a single dose and found no improvement in conferring tolerance by dividing the dose of antigen over the first few days. However as both were working with doses of antigen much larger than the equivalent of 10 mg in the chicken their negative results are perhpas not surprising. Whether or not the interpretation given above of the potential effect of a divided dose is valid there does appear to be a real case for further investigation of a possible threshold level of antigen required to instate tolerance in the young animal. This interpretation is consistent with the finding that as the animal matures it becomes more difficult to make it unresponsive for long periods by the injection of protein antigens (cf. Dixon & Maurer, 1955, Smith & Bridges, 1959), presumably because there is a quantitatively greater number and range of available antibody-forming cells in the adult animal.

The later studies on unresponsiveness described in this section were conducted with BSA as the antigen, but unresponsiveness to this protein was not so easily brought about as in the case of BGG. A total dose of 200 mg in the first 8 days

of life was insufficient to induce an unresponsive state that could last for 11 weeks, though a dose of 600 mg or more could do this. The results of the experiments with BSA drew attention to the correlation between the size of the initial injections and the duration of ensuing unresponsiveness, being in this respect consistent with the observations of Wolfe et al. (1957), and to the ability of challenge injections to prolong the non-reative state in animals already fully unresponsive. The same conclusions have been drawn from the results of work done in the rabbit (Smith & Bridges, 1958) and the mouse (Terres & Hughes, 1959; Dresser, 1961).

The duration of unresponsiveness being finite it is possible to make an estimate of the number of molecules of antigen remaining in the circulation at the time when unresponsiveness ends. Smith and Bridges (1959) obtained a figure of 10^{12} molecules of BSA in rabbits, Humphrey (1959) one of 10^{10} for HSA in the same experimental animal, while Dresser (1961) using BGG, found that mice retaining 10^{10} molecules were tolerant whereas when only 10^{7} molecules remained these same mice were partially immune. From the antigen elimination studies carried out with ^{131}I-BSA in the chicken the half-life for this protein appears to be approximately 24 hours. Using this figure and taking the molecular weight of BSA as 70,000 (100 mg $\sim$ 10^{18} molecules) a dose of 100 mg given to a chicken will be virtually eliminated in 60 days assuming a continuous catabolic rate of removal. The absence of any precise definition of the duration of unresponsiveness in the various groups in experiment T/5 makes it difficult to be certain of the actual number of molecules remaining at the time of breakdown of tolerance, but group B (6 x 100 mg) seems most suited for the calculation. At both the 11 and 16 week challenges the

group gave a partially unresponsive type of reaction, a number of individual birds being capable of an immune response while others were not. It appeared therefore that in this group tolerance was about to break down. By calculation it was found that at the time of the first challenge at 11 weeks group B birds had only about 10^8 molecules of antigen remaining in the circulation. The further injection of c. 50 mg BSA at this time would have supplied sufficient additional molecules to maintain the level above c. 10^7 for a further 35 days which was when the second challenge was given. Although these calculations can be no more than approximate they do at least indicate that at the time when tolerance is breaking down the number of molecules of antigen remaining in the circulation of chickens is of the same order of magnitude as those determined for rabbits and mice.

Earlier in this discussion it was suggested that the reason for the difficulty in making the chicken unresponsive to BSA by post-hatching injections was the fact that it is an extremely efficient producer of precipitins against this serum protein. There may be another more simple explanation. Since the unresponsive state appears to be intimately associated with, if not entirely dependent upon, the persistence of a critical minimum level of antigen in the body it is to be expected that the more rapid the elimination of an injected antigen the shorter time does the animal remain unresponsive. Duration of unresponsiveness then becomes a function of the half life of the antigen in the experimental animal being used. Smith and Bridges (1959) found the half life of BSA in young rabbits to be between 2 and 3.5 days while, as mentioned earlier, the half life of BSA in the young chicken is about 24 hours which is consistent with the generally higher metabolic rate of avian species compared to mammals. Clearly then the chicken will eliminate

a given quantity of antigen in a much shorter time than will the rabbit and an induced unresponsiveness in the fowl will be the less durable.

This argument can be more forcibly put by considering it in quantitative terms. Figure 9 shows a graph on a semi-log plot of the comparative elimination curves for an antigen such as BSA in the young chicken and the young rabbit. In constructing the graph a half life of 48 hours in the rabbit and of 24 hours in the chicken in respect of the antigen have been used. Elimination of the antigen is assumed to be catabolic throughout. Suppose that the chicken has been given 100 mg on hatching and a pair of rabbits given 10 mg and 1 mg respectively at birth and that they are all to be challenged at an arbitrary time of 5 weeks. The amount of antigen remaining at 5 weeks from the original injections can be determined from the graph by inspection. In this way it can be seen that the amount of antigen remaining in the circulation in a chicken given 100 mg at hatching is approximately 10^{-9} mg while that in the rabbit given 10 mg at birth is c. 10^{-4}mg. In the rabbit given only 1 mg at birth the amount remaining at 5 weeks, 10^{-5}mg, is still far in excess of that in the chicken, and the longer the interval between the tolerance inducing dose of antigen and challenge the greater is the difference in the amounts remaining in the two species. Thus, for a standard dose of neonatally injected antigen such as BSA, the chances of obtaining unresponsiveness at a given time of challenge are heavily biased in favour of the rabbit.

The more rapid elimination of antigen by the chicken provides a logical and satisfactory explanation of the difficulty of inducing unresponsiveness in the fowl compared to the rabbit and accounts for the need to give such very

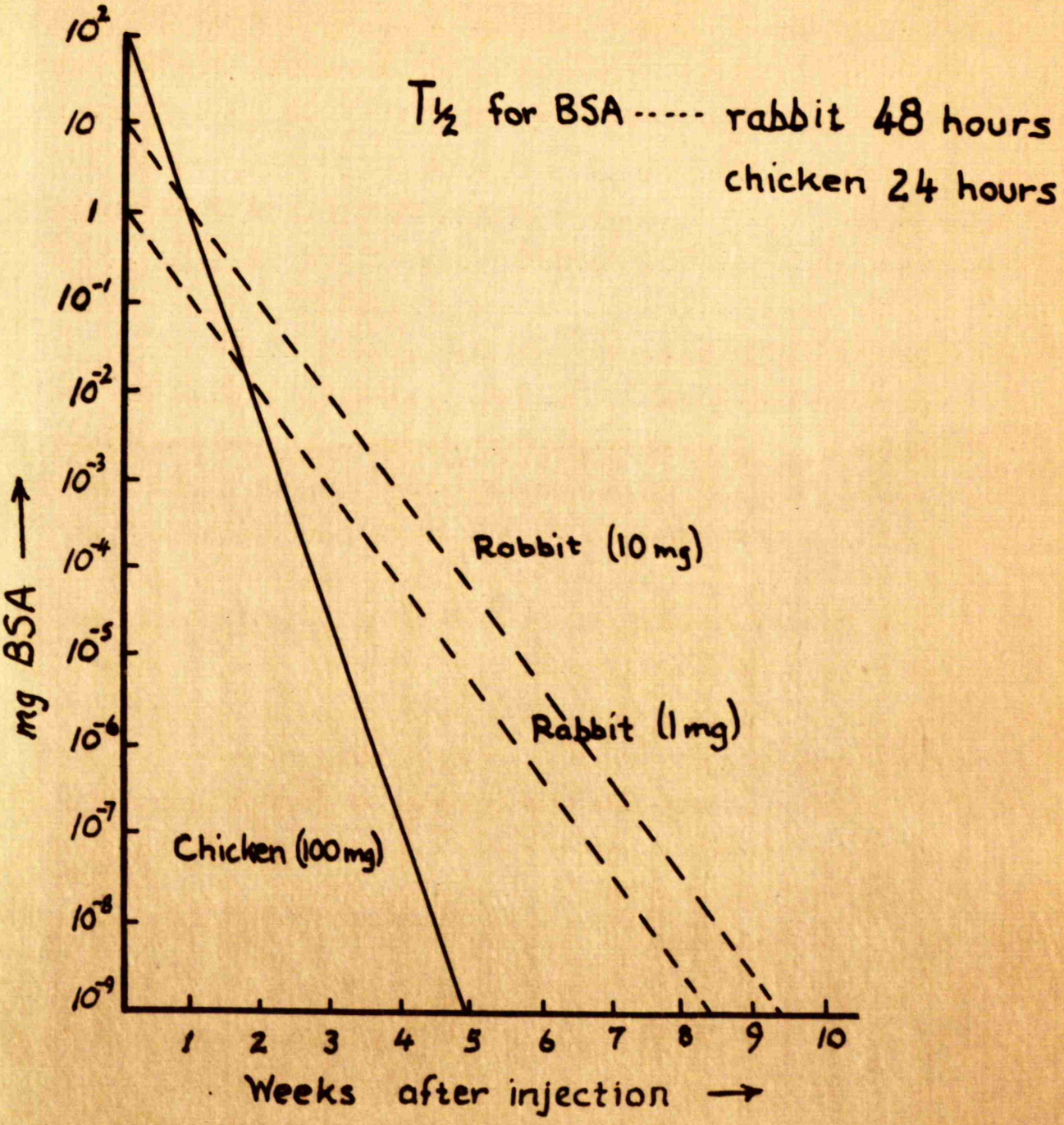


Fig. 9. Semi logarithmic plot of the elimination of BSA in a chicken given 100 mg at hatching and in rabbits given 10 mg and 1 mg at birth

large doses of antigen to baby chicks in order to induce unresponsiveness of any duration. This explanation can also account for the fact that in any one species of animal tolerance generally is more readily induced using antigens taxonomically related to the animal than by employing 'foreign' material, for the more foreign the antigen the more rapidly will it be eliminated.

While this explanation is attractive on account of its simplicity there is clearly a need for a critical quantitative examination of the induction and duration of unresponsiveness by a standard antigen in rabbits and in chickens, in a type of experiment where the two species can be used and tested side by side, and accurate determinations made of the half life of the antigen for each species. There is also a need for an investigation in one species of the correlation between the half lives of proteins of varying degrees of taxonomic relationship and the ease with these antigens can induce unresponsiveness. It is thought that an approach of this nature to the study of unresponsiveness, if adequately quantitative, might produce results that would be of considerable theoretical interest.

It is common experience in experiments with soluble protein antigens to find a considerable range in the degree of unresponsiveness shown by a group of animals receiving the same treatment providing a striking example of the observation that tolerance is not an all-or-none phenomenon. The status of the partially tolerant animal, one where unresponsiveness is incomplete and which is capable of some degree of immune response, even though it be only feeble, is not easy to determine, but the reason for its incomplete unresponsiveness is in all liklihood intimately concerned with the mechanism of unresponsiveness itself, acting at

cellular level. It can be appreciated that to obtain an absolute unresponsiveness all cells capable of forming antibody to the antigen must be suppressed in this respect but it is not yet known whether in the partially unresponsive animal all cells are incompletely suppressed or whether while most cells are inhibited a small number retain unimpaired their faculty for producing an immune response (Medawar, 1961). Thus the partially unresponsive animal occupies an important position in relation to tolerance, and has useful potential in quantitative studies of the unresponsive state.

The importance and urgency of quantitative studies of the phenomenon of tolerance has been stressed earlier in this thesis. To date the only animals in which any real attempt has been made at such quantitative assessment are the rabbit, chicken and mouse, using soluble protein antigens. From these studies have emerged the general findings that the duration of unresponsiveness is finite and related to the amount of antigen initially given, that persistence of antigen within the animal at a threshold level is essential to the maintenance of the tolerant state and that, once induced, unresponsiveness can be maintained by reinforcing injections of the antigen. Further work remains to be done, particularly with regard to an examination of the relationship between the tolerant state which is produced by exposure to the antigen in early life and the unresponsiveness induced in an immunologically mature animal by massive doses of antigen. This problem is an important one that may be amenable to at least partial solution by a study of the graph relating the amount of antigen required to be given at different ages from the neonatal period to immunological maturity to induce a standard unresponsiveness. An analysis of this type carried out in various species could perhaps

show the relative susceptibility of the animal at different ages to the influence of an excessive amount of antigen and bring to light the more obvious species differences in this respect. Figure 10 shows the general type of information which is envisaged from such studies.

One problem that immediately presents itself is how to measure a standard degree of unresponsiveness. Attempts to measure it in terms of the length of time for which the animal will remain unresponsive are fraught with difficulty in that the challenge dose of antigen may in some cases reinforce and prolong the state of unresponsiveness. It might be better to attempt a measurement in terms of a 'partial response.' For example, if groups of presumptively unresponsive and normal control animals are challenged with antigen at the same time it is possible to work out a percentage quotient $\frac{RT}{RC} \times 100$ where RC is the measured antibody response of controls and RT that of the partially unresponsive animals. This would vary from 100 in the case of animals showing no unresponsiveness to 0 for complete unresponsiveness with regard to one particular antigen. In this way it would be possible to establish a relative end-point for measuring a standard degree of unresponsiveness, such as a magnitude of response half that of controls.

A plot of the percent response against amount of antigen given in early life is shown in Figure 11 for the results of the challenges at 11 and 16 weeks in experiment T/5. The results show remarkable regularity. It might have been expected that in this system the curve would approach the antigen axis in a more asymptotic way, but in the experiment the tolerance inducing dose of antigen was given as divided doses and this may in part account for the straight line character of the curve. On the other hand the elimination of

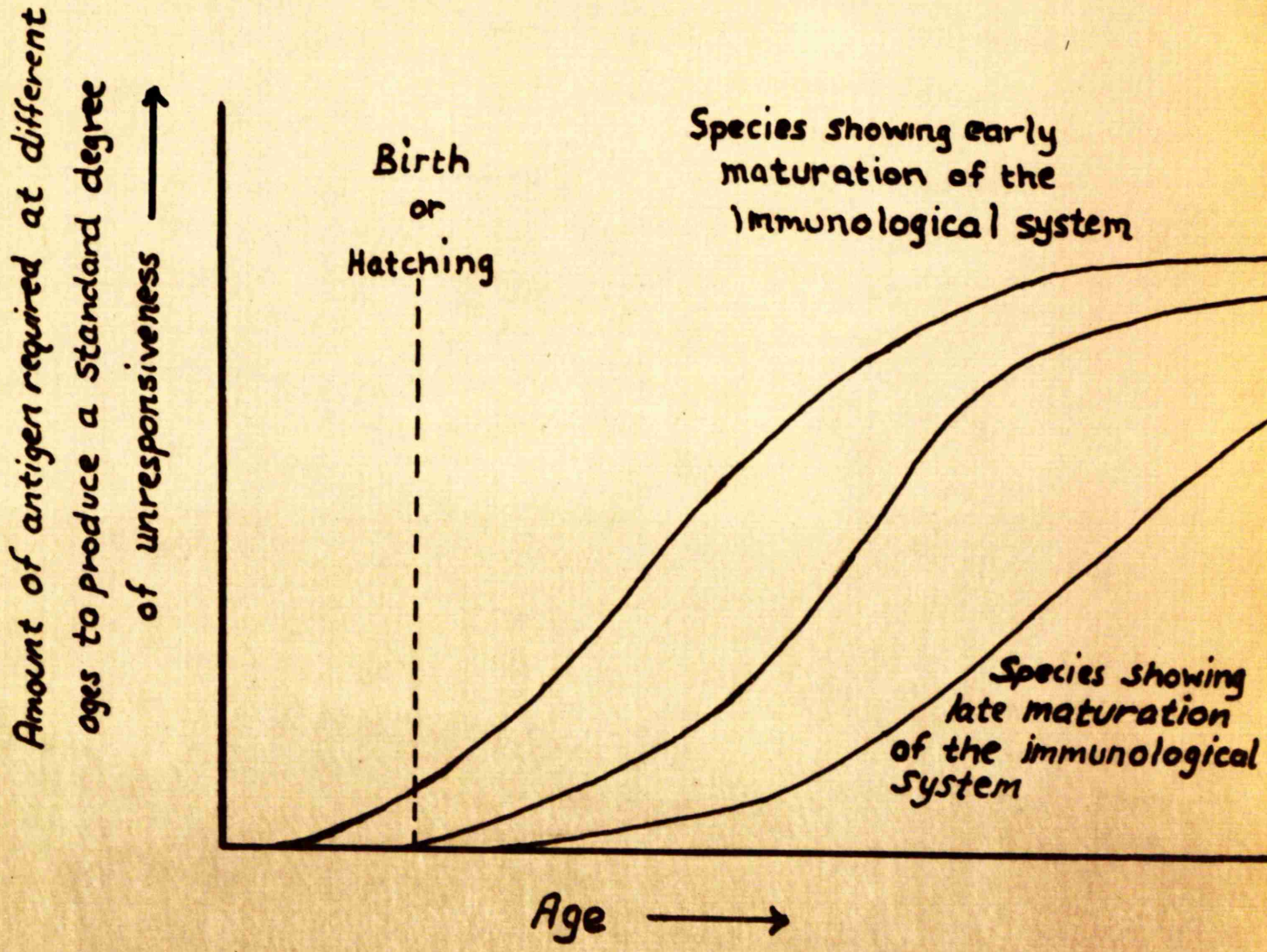


Fig. 10. *Graph showing the type of relationship that might be expected to hold between the age of a young animal and the amount of antigen required to produce a standard degree of unresponsiveness.*

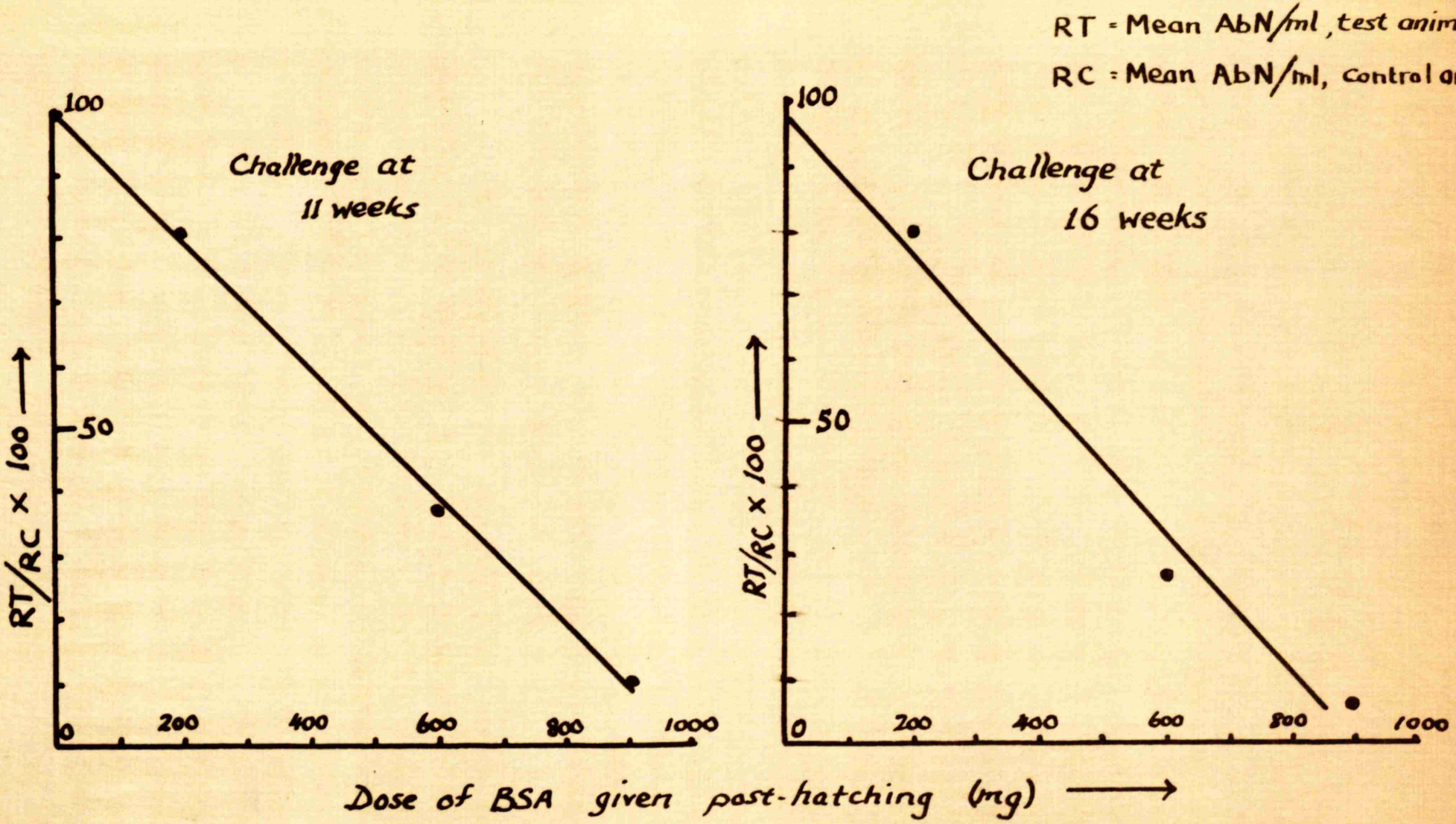


Fig. 11. *Relationship between the size of the post-hatching dose of BSA and the antibody response resulting from a challenge with BSA at 11 weeks and at 16 weeks.*

antigen is so very rapid in the fowl that it is hard to reconcile the very minute quantities of antigen left at challenge with the effect they appear to be producing. In any event it is clear that from this type of curve one can calculate a 50% end point, i.e. an amount of antigen which given to young animals will reduce the response on later challenge to one half of that of control animals. Further work along these lines is indicated so that some quantitative assessments of unresponsiveness may be made.

It would seem reasonable to attempt to measure the amount of antigen required to produce this standard amount of unresponsiveness in animals of at least one species ranging from embryos or neonates to immunologically mature animals. Such an experiment would involve very large numbers of experimental animals and controls and for this reason the chicken would seem to be a particularly suitable subject. It would probably be necessary to use an antigen less 'foreign' to the chicken than BSA in order to induce significant degrees of unresponsiveness without having to use excessively large doses of antigen.

The phenomenon of immunological tolerance, i.e. the specific inhibition of the immune response takes its place alongside the secondary response and the anamnestic response as one of the essential phenomena of immunology. It is a concept with far reaching practical and theoretical biological implications and one with applications in fields of study other than pure immunology. Indeed the potential usefulness of a method for the specific inhibition of immunological reactions has been likened to the value of enzyme inhibitors such as cyanide and iodoacetate that have been so useful in the study of cellular metabolism (Medawar, 1956). Considering the comparatively recent discovery of

the phenomenon there is already a very impressive range of knowledge about tolerance, particularly concerning qualitative aspects. The way is now open for further exploration with a more quantitative bias especially in connection with an examination of the relationship between a dose of antigen that will suffice to establish unresponsiveness and that which is required for the maintenance of the unresponsive state. Investigation of these problems is not a simple matter and it is unlikely that they will be readily solved. Quantitative studies with simple precipitin systems offer a suitable means of approach to a number of these problems and will probably play a significant part in providing some of the solutions.

9. SUMMARY OF SECTION

Some aspects of the state of immunological unresponsiveness to soluble protein antigens following their neonatal injection have been studied in the chicken using BGG and BSA as antigens. Unresponsiveness was assessed by challenging treated birds at various time intervals after the initial injections with ^{131}I-trace-labelled antigen and studying the rate and pattern of removal of the antigen from the circulation, and by measuring the precipitin response to the challenge injection.

It was found that unresponsiveness could be more readily achieved to BGG than to BSA. Unresponsiveness never followed the injection of up to 40 mg of antigen into the 14 - day old embryo but could be brought about by serial post-hatching doses. For BGG it was found that small amounts (10 mg) were more effective in inducing unresponsiveness if given as divided doses in the early post-hatching period rather than if given as a single injection on the day of hatching. No such potentiating effect was observed when large amounts (50 mg) were given as divided doses. These findings suggest that it is important to maintain a minimum level of antigen in the circulation over a period of at least a few days when attempting to induce unresponsiveness.

With BSA greater amounts of protein were required for the satisfactory induction of unresponsiveness. With a total of 900 mg as 9 doses of 100 mg at weekly intervals from hatching the majority of birds were capable of a normal antibody response at 26 weeks though failing to respond to challenge at 11 and 16 weeks. However, 2 birds so treated were fully unresponsive at 26 weeks and the sole survivor again failed to produce antibody at 38 weeks.

The results verify the general observations that the duration of unresponsiveness is related to the amount of antigen given in early life to induce it, and that the maintenance of the unresponsive state depends upon persistence of antigen. The results also draw attention to the greater difficulty of making chickens unresponsive to BSA compared to the rabbit and mouse. This is considered to be due either to the chicken's particular ability to produce precipitins to this antigen or, more simply, to the very rapid elimination of BSA by the chicken.

The need for more quantitative studies is stressed and a method that is considered suitable for the measurement of a standard degree of unresponsiveness is proposed. The chicken is suggested as a useful experimental animal for the type of quantitative studies envisaged.

Also described in this section is one experiment carried out on pigs. The administration of 500 mg BGG within 12 hours of birth was unable to bring about a reduced response to challenge with the same antigen some 10 weeks later. The desirability of investigating the phenomenon of immunological tolerance in as many species as possible is emphasised.

SECTION F

STUDIES ON IMMUNOLOGICAL UNRESPONSIVNESS: THE EFFECT OF 6-MERCAPTOPURINE ON PRECIPITIN PRODUCTION IN THE DOMESTIC FOWL

I. INTRODUCTION

Having demonstrated that, compared to small laboratory mammals such as the rabbit, mouse and guinea pig, the domestic fowl is not a subject in which a long-lasting immunologically unresponsive state can be readily established by the conventional technique of contact with antigen in early life, it was felt to be of interest to determine whether this species was susceptible to antibody suppression by other means. Among the most commonly used agents for suppressing antibody synthesis are cortisone and related compounds, but as their effect is apparently mediated by a depression of the body's lymphoid and reticulo-endothelial systems, the suppression is generalised and does not relate to any particular antigen. The same is true of the suppressing effects of X-irradiation. At the time when the experiments to be described were undertaken there was a current interest in a cytotoxic drug which had been found capable of depressing antibody formation. The drug in question is a purine analogue, 6-mercaptopurine (6-MP), that, according to the reports of one group of workers, could cause a complete suppression of the primary antibody response of adult rabbits to BSA if administered at the same time as the course of antigen injections. The suppression was specific in the sense that only the antigen given along with the drug was unable to stimulate antibody formation once drug treatment ceased. The immune response to another antigen at this time was normal.

Many compounds that are analogues of the physiological purines are inhibitory anti-metabolites and can cause a depression in mitotic incidence (cf. Biesele, 1958). The 6-substituted purines are of considerable interest because of their ability to inhibit the growth of certain neoplasms

and in some cases to affect the growth of normal tissues. While the particular mode of action of these drugs cannot yet be explained in detail it is generally accepted that they interfere with nucleic acid metabolism, following their incorporation into the nucleic acid, for it is possible, under certain circumstances, to counteract their toxicity with various physiological purines, nucleosides and nucleotides. In particular, 6-MP is presumed to act as an "anti-purine" i.e. to interfere with nucleic acid biosynthesis along one or more of the pathways concerned with purine metabolism (Elion & Hitchings, 1957) 6-MP does not possess the same degree of tissue toxicity that is shown by some of the other purine analogues, and is used therapeutically in the treatment of acute leukaemia in both children and adults, and in chronic myeloid leukaemia (Lewis 1960). Periods of remission are more frequently obtained in children and may last up to six months. It causes a marked fall in blood granulocytes, platelets and reticulocytes because of its action on the bone marrow but it does not depress the lymph nodes.

The first description of the use of 6-MP in depressing the immune response was that of Schwartz, Stack & Dameshek (1958) who studied the effect of the drug on the antibody response of rabbits hyperimmunised with BSA. They found that when the drug was given concomitantly with the course of antigen injections it successfully suppressed the formation of antibody. When the drug treatment was carried out in the period preceding immunisation very little depression was obtained, and once antibody formation had commenced the administration of

6-MP exerted no influence on the amount of antibody in the serum. Later work (Schwartz, Eisner & Dameshek, 1959) demonstrated that whereas 6-MP could block completely the primary response to purified protein antigen its effect on the secondary response was minimal. It was further observed that the antibody suppression was of a highly specific nature, for rabbits rendered unresponsive to HSA by this drug and remaining refractory to that antigen throughout a second and third challenge, were simultaneously capable of producing a normal primary and secondary response to BGG (Schwartz & Dameshek, 1959). Because of evidence that the inhibitory effect of 6-MP on antibody formation occurs during the induction phase that follows antigenic stimulation, and is of a specific nature, it was claimed that the use of this drug provided the means for inducing a state of true immunological tolerance in adult animals.

This new approach to the problem of achieving an inhibition of the antibody response was soon adopted by several workers and within a short time there appeared a number of reports concerning the effectiveness of 6-MP in depressing immune response in various immunological systems. Meeker and colleagues (Meeker, Condie, Weiner, Varco & Good, 1959) recorded a prolonged survival of skin homografts in rabbits treated with 6-MP and Calne (1960) found that the rejection of renal homografts was modified in dogs to which he had administered the drug. The use of 6-MP enabled Hoyer, Condie and Good (1960) to delay the onset of experimental allergie encephalomyelitis (EAE) in rabbits, and Berenbaum (1960) found that 6-MP brought about a reduced response to TAB vaccine in mice.

Further work with BSA in rabbits reported by La Plante and Condie (1960) has indicated that suppression of the secondary antibody response can be achieved if the dosage of the drug is raised to 12-15 mg/Kg/day.

Not all reports were of positive results, however, and in at least two cases 6-MP was recorded as being ineffectual in producing prolonged unresponsiveness in rabbits to soluble proteins (Hoyer, Good & Condie, 1960; Robinson & Christian, 1960), but in both these cases the antigen was administered in Freund's adjuvant and therefore provided a much stronger antigenic stimulus than the single intravenous injection of Schwartz and Dameshek. An unsuccessful attempt was made by the administration of 6-MP in the post-operative period to subdue the homograft reaction in a young human patient in whom it had been necessary to carry out a non-compatible renal transplant. In this particular instance it was found that full benefit could not be derived from 6-MP because of its inherent toxicity for the young and already weakly patient.

The experiments which follow were carried out to study the effect of 6-MP on the immune response of the domestic fowl to soluble protein antigens.

2. EXPERIMENT 6-MP/1

This preliminary investigation was undertaken to determine whether the immune response of adult hens could be altered by 6-MP

Birds

In this experiment 20 adult hens of the Rhode Island Red variety were used. They were culls from a commercial flock of laying hens that had completed one season's laying and had been found to be of lower than average productivity. All birds were in good health but inclined to be fat. During the two months preceding this experiment they were used as a source of normal chicken serum and underwent serial blood sampling two or three times weekly by a venesection technique. The last sample was taken some two weeks prior to the commencement of this experiment. Such treatment rendered the wing veins unsuitable for accurate intravenous injection and consequently in this experiment the intraperitoneal route was used for the administration of antigen.

Antigen

6.0 g of Armour's BSA was trace-labelled with ^{131}I using the ICI Method. After dialysis and centrifugation the protein concentration was 46 mg/ml and the dose level for immunisation was 1 ml/Kg.

6-Mercaptopurine

The 6-MP dose level adopted by Schwartz and colleagues for the suppression of antibody formation in the rabbit was 6 mg/Kg, given as a daily intramuscular injection for a period of 14 days. Their drug treatment

commenced on the day of immunisation and in some cases on the day before. In this experiment with adult hens it was decided to commence injecting the drug on the same day as the antigen and to continue treatment for ten days only, for by that time antibody production in the chicken has normally reached its peak.

6-MP being sparingly soluble in aqueous solution at physiological pH's it was administered as a suspension in normal saline. The suspension was prepared in bulk on the day that injections were started and was stored at $5^{o}C$. The concentration of this suspension was 6 mg/ml making the dose volume 1 ml/kg. Injections were made in the thigh muscles, right and left legs being used on alternate days. Apart from a stiffness of the leg and occasional bruising at the site of injection none of the 10 treated birds appeared to suffer any serious disturbance as a consequence of their daily treatment with the drug. They continued to be lively, to eat well, and to lay eggs throughout the period of observation.

Experimental Procedure

The 20 birds were randomly divided into two groups of 10 each. One group was treated with 6-MP and the other was an untreated control group. Both groups were immunised with ^{131}I-trace-labelled BSA and elimination of antigen was studied. Following completion of the course of treatment with 6-MP, quantitative determination of serum antibody levels were carried out.

Each of the 10 birds of the drug-treated group received an intramuscular injection of 6-MP one hour before immunisation. The trace-labelled antigen was administered by the intraperitoneal route to both groups of birds.

TABLE I

Group mean figures for elimination of ^{131}I - BSA in experiment 6-MP/1

The values given refer to activity per ml of serum, expressed as a percentage of the 19 hour level

Group	Hours after injection of antigen				
	19	67	91	115	139
Control	100	36.9	17.1	1.2	0.4
6-MP	100	32.5	18.0	3.4	1.1

Because of the distended abdomens of these laying birds, and the considerable amounts of abdominal fat, it was not easy to ensure that all the injected antigen passed freely into the peritoneal cavity, and the antigen elimination figures later obtained suggested that in at least two birds the antigen was injected into the gut lumen or into a developing egg and rapidly removed from the body.

Commencing on the day following antigen injection, and continuing at 24-hour intervals, small volumes of blood were removed from all birds so that the elimination of the injected antigen might be studied. Such sampling was carried out up to and including the 7th day after injection and in a few cases went on to the 8th and 9th days. Serum obtained from blood taken on the 10th day was used for carrying out an agar diffusion precipitin test for comparing the responses of the birds. A further sample was taken on the 13th day and used for quantitative determinations of circulating antibody levels by the "percentage-antigen-precipitated" method.

Experimental Results

The group mean clearance rates of antigen are shown in Fig. 1 and in Table 1. Only 8 of the 10 birds are included in the control group. In the two cases rejected the amount of activity in the blood at 20 hours was small and subsequently remained so. It was considered that this was the result of faulty injection of antigen for one of the reasons mentioned earlier.

The pattern of antigen elimination for the 8 controls was normal. A less uniform picture was presented by the drug-treated birds. Their antigen elimination curves showed a greater scatter and lacked a well-defined distinction

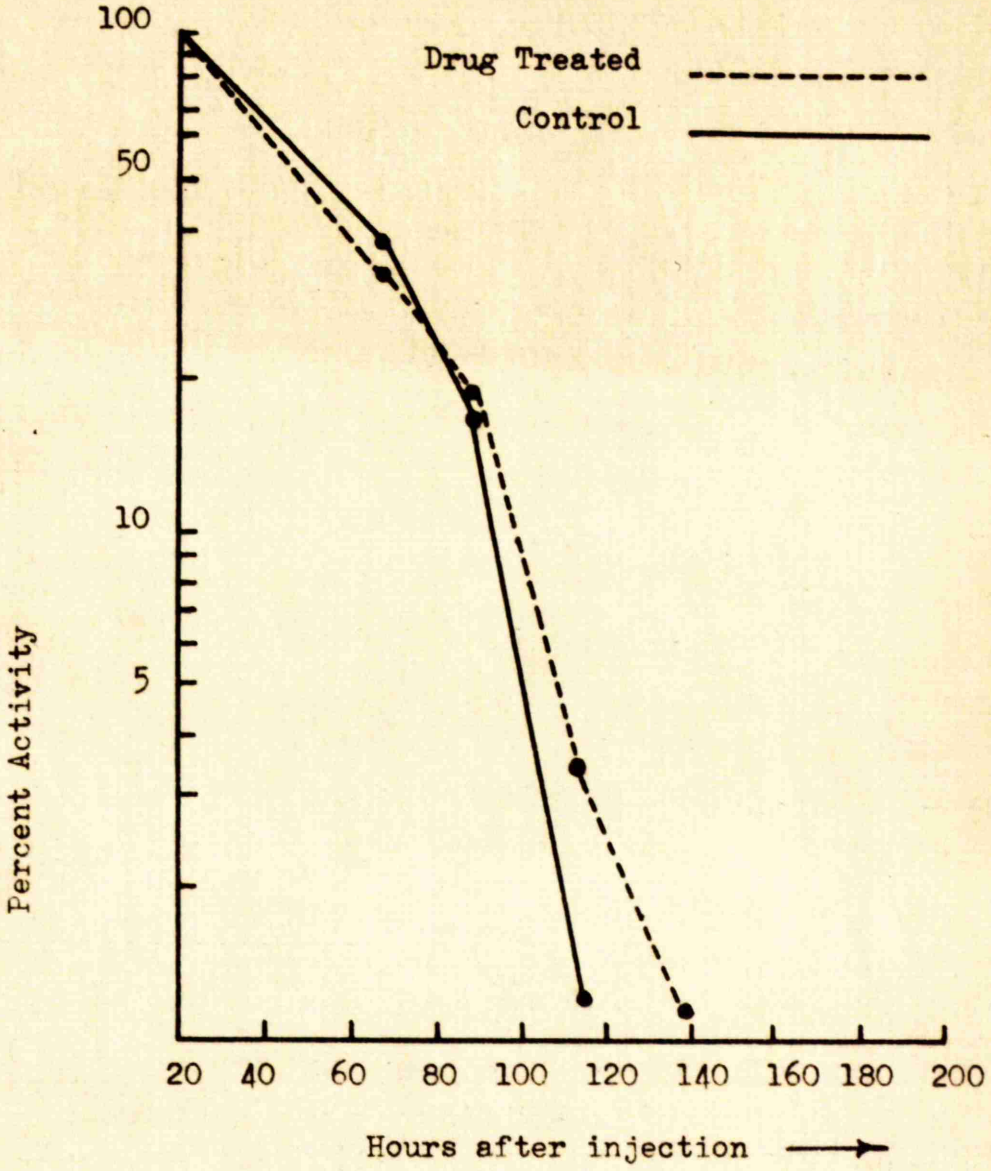


Fig. 1. Group mean patterns of antigen elimination in experiment 6-MP/1.

TABLE 2.

Antibody responses of control birds and of birds treated with 6-MP.

Control Sera			6-MP Sera		
No.	10th Day (Agar diff)	13th Day (µg AbN/ml)	No.	10th Day (Agar diff)	13th Day (µg AbN/ml)
1	+ +	82	1	-	< 30
2	+ + +	142	2	+ +	148
3	+	83	3	-	< 30
4	+ + +	150	4	+ +	61
5	+ +	121	5	-	32
6	+ + +	142	6	-	< 30
7	+	71	7	tr	127
8	+ +	146	8	tr	36
			9	tr	< 30
			10	+ +	88
Mean		117.1 ± 33.3	Mean		61.2 ± 44.7

This table records the antibody responses that developed in both groups of birds. Sera taken on the 10th day after immunisation were tested by an agar diffusion method and those obtained on the 13th day were subjected to a quantitative test.

between 'exponential' and 'immune' phases of antigen removal. However, none of the birds of this group exhibited the prolonged exponential phase of antigen clearance seen in absolutely unresponsive birds.

Serum obtained from all birds on the 10th day after antigen injection was used to carry out an agar gel diffusion precipitin test. The antisera were placed in peripheral wells round a centre well containing BSA, and the plates left in an incubator to develop. They were examined after 18 hours when it was seen that lines were forming, and again after 66 hours when the lines of precipitation had developed fully. The results are recorded in Table 2, the extent and density of the line of precipitate being indicated by the conventional method for qualitative precipitin tests. Even from these essentially qualitative results it was clear that some difference in the degree of response existed, for whereas all 8 control sera had produced evidence of precipitating ability only 3 of the 10 drug-treated birds appeared to have formed detectable quantities of antibody.

A quantitative test carried out on sera taken on the 13th day by and large confirmed the findings of the qualitative agar diffusion test. Individual and group mean serum antibody levels are given in Table 2.

The specific activity of the antigen used in the analysis of the precipitin content of the sera was such that the smallest amount of antibody that could be measured with reliable accuracy was 30 µg AbN/ml. A number of sera from the drug-treated birds gave very small precipitates beyond the limits of measurement and a figure of 30 µg AbN/ml was ascribed to them. The group mean antibody levels in

μg AbN/ml of serum were 117.1± 33.3 for controls and 61.2± 44.7 for drug-treated birds. Seven of the 10 treated birds had antibody levels below 71 μg AbN/ml which was the lowest figure in the control group. On statistical analysis ('t' test) there was found to be a highly significant difference between serum antibody levels of the two groups ($P<0.01$).

Discussion

Because the wing veins of the birds used in this experiment had been largely obliterated by repeated venesection it was found necessary to give the antigen by the intraperitoneal route. Consequently it is not possible to place much emphasis on the antigen elimination figures as indicating any difference between the two groups of animals in respect to their handling of the antigen. At best it can only be said that the drug-treated group showed a wider scatter of individual elimination curves than did the controls, and had a tendency to retain antigen for a slightly longer period. No completely unresponsive birds were detected by this technique, however.

More reliable information was obtained from the measurement of circulating antibody on day 13 and the previous agar diffusion test on day 10. In both cases a very marked difference was observed in the number of birds in each group that gave evidence of a precipitin response. Whereas all 8 controls had a readily measurable antibody level, 6 of the 10 birds that had been treated with 6-MP had a very low antibody level on the 13th day, and the other 4 had normal antibody levels. With one exception, (No. 7 of the experimental group) the results of the agar diffusion test carried out on serum obtained on the 10th

day were in general agreement with those of the quantitative test using serum taken 3 days later, which indicated that the antibody response of the drug-treated birds was actually suppressed and not merely delayed. No attempt was made to determine whether the failure of response was a specific or general phenomenon.

While no absolute suppression of antibody formation was attained in this experiment the results, based on serum antibody levels at 10 and 13 days after injection of antigen, were sufficiently definite to encourage further work using 6-MP.

3. EXPERIMENT 6-MP/2

This experiment differed from the previous one in a number of ways. The changes were made in order to eliminate some of the objectionable features of the preliminary experiment and to work under conditions similar to those used by Schwartz and Dameshek.

(i) The birds used were of the standard Golden Legbar cross.

(ii) The antigen was administered by the intravenous route, a more satisfactory procedure from the point of view of studying antigen elimination patterns.

(iii) The 6-MP was injected in actual solution as used by Schwartz and Dameshek.

Birds

So that it would be possible to compare the effect of 6-MP on antibody production with the type of unresponsiveness resulting from intensive post-hatching injections of antigen, the birds used were of the Golden Legbar x Light Sussex variety. As in the first 6-MP experiment adult hens (15 months) culled from a commercial flock were used. For the duration of the experiment they were maintained in tiered fattening cages and fed an ad lib. diet of layers' mash.

Antigen

The antigen was again Armour's BSA trace-labelled with ^{131}I by the ICI method of preparation. The stock solution contained 28 mg protein/ml and a dose level of 40 mg/Kg was employed.

6-Mercaptopurine

In this experiment the 6-MP was not in saline suspension but was prepared in solution as recommended by

Schwartz, Eisner and Dameshek (1959) by dissolving each 100 mg of 6-MP in 1.0 ml of N-NaOH and diluting with normal saline to the required concentration. Each day's solution was freshly prepared and diluted to 6 mg/ml, giving an effective dose volume of 1 ml/Kg.

Experimental Procedure

The 22 birds were randomly divided into two groups of 11 each and the experiment was conducted on the same lines as previously, with the exception that the first injection of 6-MP was given 24 hours prior to the intravenous administration of labelled antigen. The rate of clearance of antigen was determined by measuring the radioactivity of serial blood samples taken at 24-hour intervals.

Commencing on the 5th day after injection of antigen and continuing until the 9th day, serum was obtained for serological tests.

Day-to-day qualitative tests for the presence of antibody were made using agar diffusion methods. A final sample was taken on the 12th day to verify that antibody levels were waning and that there was no delayed antibody response from the drug-treated birds. Quantitative determination of circulating precipitin levels was carried out on the 8th day's sample, using ^{131}I-trace-labelled antigen in the "percentage-antigen-precipitated" method.

All birds were weighed at intervals before, during and after the experiment to see whether 6-MP was adversely affecting general bodily condition.

TABLE 3.

Group mean figures for elimination of ^{131}I - BSA in experiment 6-MP/2.

The values given refer to activity per ml of serum expressed as a percentage of the 19 hour level

Group	Hours after injection of antigen				
	19	67	91	115	139
Control	100	32	16.1	1.2	0.4
6-MP	100	32.5	18.0	3.4	1.1

Experimental Results

Group mean antigen elimination patterns were not very different from one another although there was a slightly greater persistence of antigen in the 6-MP group (see Table 3). In the control group elimination of antigen took the usual course, with a well defined phase of accelerated elimination occurring between 90 and 115 hours after injection of the antigen. All but 2 of the hens that had been given 6-MP showed a similar clearance of antigen, the immune elimination phase being well characterised. Of the two exceptions one bird had a delayed immune phase and did develop antibody, while the other lacked a well defined acceleration of antigen clearance and no precipitating antibodies could be detected in its serum.

TABLE 4

Serum antibody levels 8 days after injection of antigen

Control Group		6-MP Group	
Bird No.	µg AbN/ml	Bird No.	µg AbN/ml
1	279	12	431
2	294	13	477
3	208	14	537
4	393	15	134
5	241	16	0
6	303	17	205
7	155	18	190
8	233	19	252
9	243	20	132
10	137	21	258
11	288	22	450
Range	137 - 393	Range	0 - 537
Mean	252	Mean	278
S.D.	± 72	S.D.	± 171

In a few birds antibody was detected on the 5th day following antigen injection, and by the 6th day all but 5 were giving good precipitin responses on the agar plate test. One of these 5 birds was in the control group. It failed at all times to give a positive result on agar diffusion but was found to contain 155 µg AbN/ml when quantitatively tested. The 4 birds of the 6-MP group that failed to respond on the 6th day were the ones whose elimination of antigen was delayed and in which the immune drop was not very clearly defined. Antibody was produced in due course by 3 of these hens, while the fourth failed completely to do so, up to and including the 12th day after antigenic stimulation.

The quantitative determinations of antibody present in the sera 8 days after antigen injection showed that there was very little difference between the groups (see Table 4). Excluding the one negative response in the 6-MP treated hens it was found that the range of antibody levels was very similar in the two groups. Highest levels were in fact obtained from the treated birds and not from controls, but the response by the control group was the more uniform. Mean values for all animals in each group, control and experimental, were 251.9 $\pm$ 74.7 and 278.7 $\pm$ 171.0 µg AbN/ml of serum. These two levels were not statistically different.

Discussion

The results obtained in this experiment did not confirm those of the preliminary investigation with 6-MP. On the contrary there was virtually no difference in the immunological behaviour of the two groups of animals. Only two of the treated birds retained antigen for a period that was greater than that observed in the control group.

One of these had a delayed immune phase in the antigen elimination pattern and did produce antibody, but in the other bird neither an immune phase nor antibody development was observed. Any difference between the two groups, and thus any influence exerted by the 6-MP, must rest on the lack of immune response by this one bird, and this is outwith the limits of significance. Had the lack of response in this bird been due to treatment with 6-MP it would have been expected that some at least of the other 10 members of the group would give evidence of a reduced antibody response. This was not so, and several of the birds gave very high antibody levels on the 8th day. Because non-responding animals are known to occur naturally it is even more difficult to ascribe the one recorded negative response as being the result of drug treatment. It is not certain that the matter could have been elucidated by a test for the specificity of the unresponsiveness using another antigen, and as the implication of the group result was quite clearly that the use of 6-MP under the conditions described had not produced a state of immunological unresponsiveness, the matter was not taken further.

Between this experiment and the previous one, there were a number of points of difference. In the preliminary experiment the drug was administered in the form of a suspension in normal saline, whereas the birds treated with 6-MP in this second experiment received the drug in an alkaline solution. For reasons already mentioned, it was necessary to use the intraperitoneal route of antigen injection in the case of the first experiment instead of the usual intravenous route. Finally there was the difference between the two breeds of animal used that had to be considered.

Whether any single factor, or any combination of two or more, was responsible for the conflicting results is difficult to assess. The procedure employed in the second experiment, both as concerns 6-MP and the route of immunisation, conformed more closely to the conditions of the experiments of Schwartz et al, but this was the one in which 6-MP was without effect on the immune response. It was possible that the drug in solution form was unsuited for this purpose in the chicken, or that the dosage of 6 mg/Kg, known to be adequate for the rabbit, was insufficient for the chicken which is a more vigorous producer of precipitating antibody.

The second tentative explanation was to some extent corroborated by the apparent lack of toxicity of the drug for the chicken. Most workers who have made use of 6-MP in rabbits have recorded toxic symptoms in their experimental animals, usually shown by a loss of weight, poor appearance and diarrhoea, and, in some cases, death of the animal. The adult hens used in both experiments maintained good condition throughout the period of observation, though the number of injections of 6-MP was smaller since the course of treatment lasted 10 days only and not 14 as in the rabbit. During the second experiment all birds were weighed three times, on the 1st and 10th days of 6-MP injections and again 5 days after the final injection. Comparing the weights on days 10 and 15 with those on day 1 it was found that a small and equal weight loss occurred in both groups that could largely be explained by the fact that no overnight starvation preceded the weighing on day 1, and by the repeated handling and blood sampling to which the birds were subjected during the

TABLE 5.

Variation in body weight during the experiment

Group	10 Days		15 Days	
	Range	Mean	Range	Mean
Control	68.7 - 97.6	90.7	78.2 - 101.1	92.9
6-MP	83.7 - 104.1	91.8	78.2 - 116.8	92.3

Variation in body weights recorded on day 10 and day 15 compared to body weight on day 1 (1st injection of 6 - MP) as 100%. The variation in the 6 - MP group was quite clearly no different from that taking place in the control group.

experiment. Table 5 gives the type of body weight variation that was recorded. The amount of variation was the same in both groups.

Because of the relatively short time that it takes the fowl to produce considerable quantities of antibody compared to the rabbit, it was felt that the possibility of the need for a higher dose level of 6-MP than 6 mg/Kg should be investigated, and that the toxicity of the drug for the fowl should also be studied.

4. *THE EFFECT OF 6-MERCAPTOPURINE ON THE BODY WEIGHT OF ADULT FOWLS*

To determine whether adult birds were able to withstand serial injections of 6-MP at doses greater than 6mg/Kg/day, a small toxicity trial was carried out. Toxicity was assessed by daily inspection of the general health of the birds together with a measure of the rate of weight gain.

A total of 9 birds was used in this trial. Formerly, these animals were part of a control group in an experiment concerned with the induction of immunological unresponsiveness by contact with antigen during early life. They had been twice immunised with ^{131}I-BSA, once at 11 weeks and again at 16 weeks of age. On each occasion, serial blood samples were taken over a period of 7 days. Following the latter period the birds were rested for three weeks, being maintained in fattening cages and fed on an ad lib. diet of layers' pellets. When the experiment commenced all 9 were in reasonable bodily condition and were 20 weeks old. The 9 birds were split into 3 groups, of 3 birds each, designated A, B, C.

The 6-mercaptopurine was prepared daily, just before injection, according to the directions of Schwartz, Eisner & Dameshek (1959). Each 100mg of solid powdered 6-MP was dissolved in 1.0ml of N-NaOH and the solution diluted to the required concentration with 0.9% NaCl. The two dose levels employed in this trial were 10 and 20mg of 6-MP per Kg body weight, so the stock solution was prepared to contain 10mg/ml. Thus birds given the 20mg dose level (group B), received twice the volume administered to those on the 10mg dose level (group A). Because of the unphysiological nature of the solvent, in this and later experiments control groups injected with solvent alone were always included. The control group (group C) was

given a daily injection of the NaOH-NaCl solvent at a volume dosage equivalent to that of group B. All injections were made into the thigh muscles, right and left legs being used alternately. Other sites such as the pectoral group of muscles were tried but were unsuccessful as injections in these areas caused considerable pain.

For 6 days prior to initiation of treatment, the birds were weighed each morning following an overnight starvation period of some 15-16 hours. The mean body weight for this period was taken as the basis for computing the daily individual dose of 6-MP. All birds were weighed daily immediately before injection, and all weighings were preceded by overnight starvation. Weighings were also carried out at 48 and 72 hours after injections ceased, and the 72 hour weight was used for comparison with the starting weight to assess the effect of 6-MP.

Only one fatality occurred, this being in group A which was on the 10mg/Kg dose level. The bird in question maintained body weight until the 7th day when it was noticeably dull, offering no resistance to handling and to weighing. It was unable to stand and remained in a squatting position. Sensation was not affected, and there was full use of the wings. Inappetance was in evidence for the bird was disinterested in food even after the 16 hour starvation period when the birds normally accepted food greedily. Progressive deterioration in general condition was observed, with anorexia and consequent weight loss, and the animal was destroyed when the experiment terminated. Macroscopic post-mortem examination disclosed no gross abnormality or incidental disease which might have

TABLE 6

The effect of 6-MP on the body weight of adult fowls

Group	6-MP dose mg/Kg	Bird No.	Bird Sex	Mean Initial Wt. in g	Final Wt. in g	Wt. Change in g	% change
		30	M	2022	1536	-486	-24 *
A	10	51	F	1873	2108	+235	12.5
		53	F	1541	1756	+215	14.0
		34	M	2102	2388	+286	13.6
B	20	35	M	2166	2449	+283	13.1
		52	F	1482	1644	+162	10.9
		33	M	1978	2319	+341	17.2
		50	F	1587	1625	+ 38	2.4
		55	F	1384	1534	+150	10.8

* This bird lost condition gradually throughout the experiment.

The initial weight was the mean of 6 weighings and the final weight was a single weighing made 72 hours after the last injection of 6-MP or solvent. Overnight starvation preceded all weighings.

accounted for the symptons presented. The loss of condition and anorexia could have been the result of an individual susceptibility to the toxic effects of 6-MP, or to some disease process not revealed at post-mortem.

In all other cases there was some slight day to day variation in recorded body weights but the general condition of the animals was maintained throughout. On the basis of the weighing made 72 hours after the last injection of 6-MP all birds showed a gain on their initial starting weight. These figures are recorded in Table 6. With the exception of one bird in the control group whose increase in weight was only 2.4%, all weight gains were considerable and fell in the range of 10.8 - 17.2% with a mean of 13.2%, which indicated that the repeated injections of 6-MP had wrought no serious harm to the majority of the birds used in the trial. This is quite different from the situation in rabbits, where a daily dose of 6mg/Kg for 14 days resulted in an average loss of 14% of initial body weight (Robinson and Christian, 1960).

As a result of these findings it was considered that a dose of 6-MP of up to 20mg/Kg could be employed in the fowl with reasonable freedom from toxic effects.

5. EXPERIMENT 6-MP/3

The purpose of this experiment was to determine whether a dosage of 6-MP greater than 6 mg/Kg might produce a suppression of precipitin production in the fowl.

Birds

For the experiment twenty birds of the Golden Legbar x Light Sussex Cross were used. They were 26 weeks old and had not previously been used for any experimental studies.

Antigen

Armour's BSA was trace-labelled with ^{131}I by the ICI method so that antigen clearance could be studied. The antigen was injected intravenously at a dose level of 40 mg/Kg.

6-Mercaptopurine

A solution of the drug in N-NaOH was prepared daily and diluted with normal saline to give a concentration of 36 mg/Kg. The daily dose of 6-MP was 18 mg/Kg, equivalent to 0.5 ml/Kg. Injections were made in the thigh muscles every day for 10 days, the injection on day 1 being given 4 hours before the intravenous administration of antigen.

Experimental Procedure

The 20 birds were divided into three groups of 6, 10 and 4 birds each, the groups being identified as I, II and III respectively. Group II was treated with 6-MP, Group III received parallel injections of the NaOH-NaCl solvent without 6-MP, and the birds of Group I acted as untreated controls.

Following the injection of antigen serial blood samples were collected at daily intervals for radioactivity

TABLE 7

Group mean figures for elimination of ^{131}I-BSA in experiment 6-MP/3.

The values given refer to activity per ml of serum expressed as a percentage of the 18 hour level.

Group	No. of birds	Treatment	Hours after injection of antigen					
			18	43	67	91	114	138
I	6	nil	100	51.8	34.9	21.9	6.7	0.9
II	10	6-MP	100	-	31.3	17.9	4.5	1.5
III	4	solvent	100	-	26.2	15.5	2.7	0.8

TABLE 8.

Individual and group mean antibody levels on days 10, 12 and 14. after antigen injection

Group	Bird No.	Days after injection of antigen 10	12	14
		µgAb N/ml		
I (control)	45	99	153	66
	46	86	78	- +
	47	275	150	71
	64	405	300	162
	65	263	140	89
	66	570	218	58
	Mean	283 ± 185	173	89
II (6-MP)	36 *	74	62	25
	37	535	358	171
	38	410	280	161
	41	158	93	44
	42	-	137	62
	57	160	149	95
	58	80	15	20
	59	117	64	20
	60	144	-	-
	62	92	28	17
	Mean	193 ± 163	132	68
III (Solvent)	48	126	149	18
	49	273	147	75
	67	353	312	-
	68	58	62	25
	Mean	203 ± 135	168	39

* *This bird had a delayed immune clearance of injected antigen.*

\+ *A dash (-) indicates that no serum was available for analysis.*

measurements so that antigen elimination could be followed. On the 10th, 12th and 14th days after injection of antigen serum was obtained for carrying out quantitative determinations of serum antibody levels by the "percentage-antigen-precipitated" method.

Experimental results

In Table 7 are shown the group mean figures for removal of antigen. It is clear that there was no significant difference between the groups in this respect. A phase of accelerated elimination was evident in all three groups after 91 hours and by 138 hours only small quantities of antigen remained in the circulation.

The results of the quantitative precipitin analyses are given in Table 8. In all three groups the level of circulating antibodies was undergoing decline by the 14th day. There was no evidence of reduced antibody levels, nor of a delayed response, in the birds treated either with 6-MP or with the solvent.

This experiment demonstrated quite clearly that in healthy young adult birds the administration of 6-MP at a dose level of 18 mg/Kg for 10 days did not alter the normal precipitin response to BSA, so providing confirmation of the results of experiment 6 - MP/2. The results of this experiment and those preceding it are discussed in the next part of this section.

6. *THE EFFECT OF 6-MERCAPTOPURINE ON PRECIPITIN PRODUCTION IN THE FOWL*

GENERAL DISCUSSION

Concerning the role of 6-MP as an inhibitor of antibody synthesis the experimental evidence indicated very definitely that in this respect it was ineffective in the fowl. A daily intramuscular injection of up to 18 mg/Kg was given on 10 consecutive days to young adult birds but failed to suppress the antibody response to a simultaneously administered protein antigen. In a limited number of individual cases a lower than normal level of serum antibody was produced, but it was by no means certain that this could be directly attributed to the influence of 6-MP.

These findings in the domestic fowl were in marked contrast to the results obtained by workers who used the rabbit as their experimental animal. In that species 6-MP was found to cause total suppression of antibody formation to protein antigen that lasted at least three months and was specific for the antigen administered during the course of treatment with 6-MP (Schwartz & Dameshek, 1959).

So far as was possible the experiments in chickens duplicated the conditions described by Schwartz and coworkers. In their initial experiments Schwartz, Stack & Dameshek (1958) found that the most reliable suppression of antibody formation followed when antigen was given on the same day as the first injection of 6-MP. When the 14-day drug treatment period was initiated some days before the animal was immunised the antibody response was merely a little delayed. Once immunisation had taken place and antibody formation was under way the administration of 6-MP could not alter the course of the antibody response. Thus in the chicken experiments the antigen injection was always made at the time of commencement of treatment with 6-MP. For the rabbit the most suitable daily dose of

6-MP was found to be 6 mg/Kg, and drug treatment was continued for 13 or 14 days by which time symptoms of toxicity were in evidence. Doses below 6 mg/Kg were not without effect and a linear relationship was found to exist between the dose of the drug the effect on the primary immune response.

The failure of 6-MP to suppress antibody formation in the chicken could thus have been simply a matter of inadequate dosage, even though the actual amount used was three times greater than that found to be effective in the rabbit. To some extent this supposition was corroborated by the absence of any symptoms of toxicity in chickens receiving 6-MP at a dose level of 18 mg/Kg/day. Although this evidence was based on daily inspection of the animals and did not involve any haematology, it was found that the rate of weight gain in treated birds was the same as that of normal controls.

To account for these findings it is necessary to make the assumption that the chicken has a better tolerance for 6-MP than has the rabbit. Why this is so is not certain but one possible explanation would be that the chicken is capable of a more efficient removal of the drug from the body. Purine metabolism in mammals is limited to the breakdown of purines derived from nucleoproteins whereas in birds all protein is broken down and nitrogen eliminated by the pathways of purine metabolism. It is conceivable therefore that the chicken, because of its heavy bias towards purine metabolism as a method of nitrogenous excretion, possesses a particularly efficient mechanism for the removal of 6-MP, so avoiding an accumulation of amounts that might interfere with antibody formation. While this can only be regarded as a tentative

explanation there are two facts that tend to support the idea behind it. Ammonia, which is toxic to mammals, is much less so for birds, reptiles and certain insects because in these latter species it is excreted in the form of uric acid, whereas in mammals it is detoxified by combination with CO_2 to form urea. The need of the chick for glycine is high, the quantities required being greater than can be synthesised by the birds themselves (Green, 1960), and this is presumed to be necessary for the detoxification of the ammonia to form uric acid.

If it is the case that the chicken can readily break down and eliminate 6-MP then it is understandable that no suppression of antibody formation was observed and that toxicity symptoms were not apparent. It may also be possible to appreciate why the first experiment with 6-MP in the fowl gave evidence of reduced antibody responses. In that experiment the physico-chemical state of the 6-MP was different - it was administered in the form of a suspension in normal saline and not as a solution. Under these circumstances it might not have been freely available for catabolism in view of its low solubility and there may have been an almost continuous presence of 6-MP in the birds during the period of immunisation. In this respect it is to be noted that Berenbaum (1960) gave 6-MP to mice as a suspension at a dose level of 150 mg/Kg and obtained a significant depression of response to TAB-vaccine.

There appears to have been no other work carried out on the fowl using 6-MP in relation to protein antigens, but Rubin (1960) has had experience of the drug in young chickens in which he was studying homograft survival times. He found that it was possible to prolong the life of homografts in 8-day old chicks treated with the drug but

the method was less successful when applied to birds of 3 weeks of age presumably due to the better immunological reactivity of the older birds (cf. Wolfe & Dilks, 1948).

It should, of course, be emphasised that the chicken is a very much better producer of precipitating antibodies than is the rabbit, a single intravenous injection of antigen resulting in the rapid production of copious quantities of precipitins. To obtain an equivalent response from rabbits would require repeated intravenous injections or a course of intramuscular injections of adjuvanted antigen. In most of their experimental studies with 6-MP Schwartz and colleagues immunised their rabbits with a single intravenous injection of antigen which is a relatively weak stimulus for this species, so that conditions may have been optimal for a demonstration of the antibody-inhibiting effect of 6-MP. It is perhaps significant that when rabbits were immunised with antigen in complete Freund's adjuvant, antibody formation, though suppressed, was not completely inhibited by 6-MP (Hoyer et al., 1960; Robinson & Christian, 1960).

This suggests an alternative explanation for the apparent partial suppression of antibody response observed in experiment 6-MP/1, viz., that the intraperitoneal route of antigen injection provides a weaker immunogenic stimulus than does the intravenous route, yielding a poorer antibody response that could be more readily inhibited by 6-MP. Although Deutsch, Nichol and Cohn (1949) obtained only feeble antibody production after a course of intraperitoneal injections of antigen, the 13th day serum antibody levels in control birds of experiment 6-MP/1 indicated that there had been a reasonably good production of antibody though the antibody levels were not quite so high as would have been anticipated in adult birds after an intravenous

injection of antigen. While in later experiments it was not possible to suppress antibody production in the chicken, even when using 18 mg 6-MP/Kg, such an effect might be demonstrable if the antigenic stimulus were reduced by using a smaller quantity of antigen for immunisation.

It would seem to be desirable to determine whether variation in the route of injection of 6-MP can exert any effect on the immunological system. The intravenous route has been employed in the rabbit for injection of NaOH solutions of the antimetabolite and the rabbits have not exhibited any more serious toxicity symptoms than normally follow intramuscular injection. In the bird particularly there would appear to be some justification for attempting this either with the solubilised form of the drug or with a very fine saline suspension. A more intimate contact with the antibody-forming cells might be achieved and the results would certainly be of interest.

7. SUMMARY AND CONCLUSIONS

The effect of a purine analogue, 6-mercaptopurine, on precipitin production in the fowl was investigated.

1. In a pilot experiment in which a suspension of the drug in normal saline was given to adult hens at a dose level of 6 mg/Kg there was partial suppression of the antibody response to intraperitoneally injected antigen.

2. When the experiment was repeated, using a different variety of bird and a solution of the drug in NaOH at the same dose level, the antibody response to an intravenous injection of antigen was unimpaired.

3. A small trial was undertaken to determine the toxicity of the drug for the fowl. It was found that adult birds could tolerate as much as 20 mg/Kg/day for 10 days without displaying symptoms of toxicity during or after this period.

4. Even when 6-MP was administered in doses of 18mg/Kg/day (three times the effective dose for rabbits) antibody production in treated birds was equal to that of untreated controls.

5. Reasons for the failure of 6-MP to suppress antibody formation in the chicken are discussed. It is considered that this may be due to the particularly vigorous production of precipitins by this species or to a more rapid catabolism and excretion of the drug by the fowl compared to the rabbit.

If the dose of the drug were increased or if the antigenic stimulus were weakened it might be possible to suppress antibody formation in the fowl by this method. The physico-chemical state of the drug and its route of administration remain unknown factors in determining the efficacy of 6-MP as an antibody inhibitor.

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