Immunology, 1966, **11**, 103.

The Nature of Antibodies to Goat Erythrocytes in the Developing Chicken

J. J. DELHANTY* AND J. B. SOLOMON†

Chester Beatty Research Institute, Institute of Cancer Research, Fulham Road, London

(*Received* 22*nd October* 1965)

Summary. The maturation of the immune response to goat erythrocytes in chicken from hatching onwards is described. Agglutinins in immune sera were examined for their sedimentation properties, heat stability at 65°, resistance to 2-mercaptoethanol (ME) and ability to agglutinate at 4° or 37°. Large doses of goat erythrocytes were given to favour the induction of the IgG response. Agglutinins were tentatively classified as class I, which were cold and warm agglutinins, IgM, ME-susceptible and heat-labile; class II, a warm agglutinin, IgG, ME-resistant and only slightly heat-labile; and class III, a cold agglutinin, possibly IgA, partially resistant to ME but completely heat stable. Immune agglutinin was first detected in sera from 11-day-old chicks and all three types were detectable at 17 days of age. Multiple injections of goat erythrocytes induced an earlier response than single injections. Agglutination titres, in response to weight-adjusted doses of antigen, increased from 5 to 24 weeks of age. Chicks could be made hyperimmune by 6 weeks of age when the proportion of class II antibody was much increased compared to that in primary sera.

INTRODUCTION

The pattern of antibody formation following a single injection of antigen is generally an initial production of IgM (19S) antibody followed by the later appearance of IgG (7S) antibody (Bauer and Stavitsky, 1961). Young animals might be expected to produce antibodies soon after immunization that are typical of an incompletely developed lymphoid cell system. This idea has been supported by some earlier work in which the primary response of young rabbits to sheep erythrocytes (Říha, 1962) and newborn children to *S. typhi* vaccine (Smith, 1960) was exclusively IgM antibody. More recent work has shown that the appearance of IgG antibodies may be more delayed in the young than in adults (Smith and Eitzman, 1964). However, much depends upon the nature of the immunogen (Nossal, Ada and Austin, 1964) and we have shown, in the present work, that IgG antibodies to goat erythrocytes can appear almost as soon as IgM antibodies in young chicks.

After immunization of the chick embryo, opsonizing antibody to goat erythrocytes first appears at hatching (Solomon, 1966a). The method of measuring opsonizing antibody by antigen clearance has the great advantage of sensitivity, but yields no information on the heterogeneity of the antibodies produced. We have had to resort to haemagglutination in order to follow the maturation of the immune response to goat erythrocytes in the developing chicken. Unfortunately, this method is biased in favour of the detection of IgM

* Present address: The Middlesex Hospital Medical School, Institute of Clinical Research, 40 Hanson Street, London, W.1.

† Present address: Department of Microbiology, University of Adelaide, South Australia.

agglutinins. This has been partly offset by giving enormous doses of antigen, a condition known to favour the production of IgG antibody (Uhr, 1964). Four different types of antibody are produced, two IgM, possibly an IgA and an IgG, in the response of young and adult chickens; these are classified according to their sedimentation properties, resistance to 2-mercaptoethanol, heat stability and ability to agglutinate at 4° or 37°.

METHODS

The preparation of suspensions of goat erythrocytes has been described (Solomon, 1966b). Suspensions of goat erythrocytes used for titrations were made in normal saline at a concentration of 0·05 per cent (v/v) packed cells. White Leghorn chickens were injected intravenously and later bled from a wing vein. Young chicks were injected intravenously as previously described (Solomon, 1966b). Unless otherwise stated, all sera were heated at 56° for 30 minutes to destroy complement. Doubling dilutions were made in tubes using, initially, 0·1 ml serum and 0·1 ml normal saline. An aliquot (0·025 ml) of each serum dilution was transferred to a well on a covered agglutination plate and 0·025 ml of the 0·05 per cent suspension of goat erythrocytes added. Separate tests were done at 37° and 4°. These agglutinins will be referred to as warm and cold respectively. Warm agglutinins were read after 2 hours and cold agglutinins after at least 4 hours and usually after 16 hours.

Treatment with 2-mercaptoethanol (ME) was done as follows: Equal volumes of serum (0·1 ml) and 0·2 M 2-mercaptoethanol (ME) were mixed in a stoppered tube and incubated at 37° for 1 hour. Agglutination tests were done in the presence of 0·05 M ME.

Sometimes the serum was heated at 65° for various periods of time in a water bath. Zero time was taken when the temperature of the serum rose to 63°. The serum was cooled at room temperature. In some cases, the heated sera were then treated with ME in the usual manner.

Adult immune serum was separated on a sucrose gradient by the method of Karthigasu, Jenkin and Turner (1964). Fractions were titrated without removal of sucrose. The titre was not affected by the presence of sucrose. Sixteen fractions were taken from the 5 ml of sucrose gradient and serum which had originally been overlaid with 1 ml serum. Only the first eleven fractions contained detectable antibody. Fractions were treated with ME in the usual manner.

RESULTS

The course of antibody production following primary, secondary and hyperimmunization was studied in one chicken. In this experiment agglutinins were measured at 22°. The primary response of a 4-week-old chicken is not exclusively IgM as low titres of ME-resistant antibody can be detected soon after the appearance of the ME-susceptible IgM antibody (Fig. 1). ME-susceptible antibody persisted for 4 weeks after the injection. A second injection of goat erythrocytes approximately 1 month later elicited a considerably higher proportion of ME-resistant antibody. Four injections given during 70–77 days after the first injection induced hyperimmunity and all the antibody was then ME-resistant.

A classification of the various agglutinins believed to have been detected in immune sera is given in Table 1. These antibodies could be partially separated on a sucrose density gradient but confirmation of the above classification must await their isolation. Adult

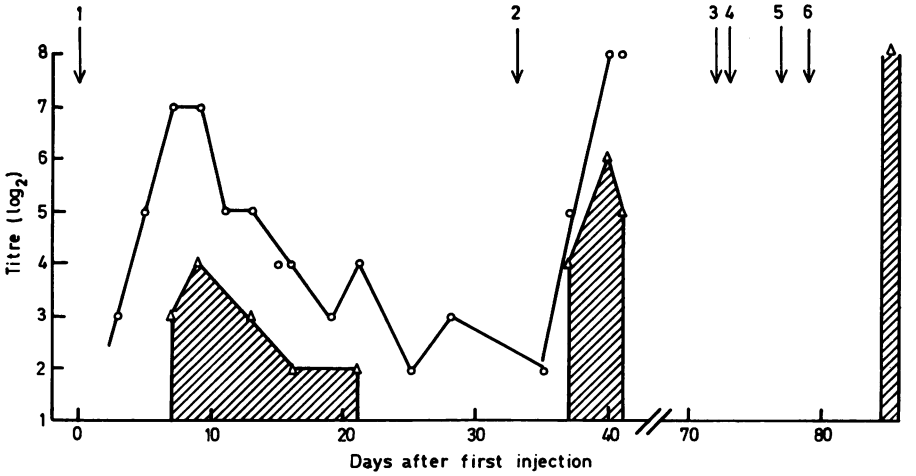

FIG. 1. Anti-goat agglutinins (22°) produced during the primary, secondary and hyperimmune response of a chicken during 1–3 months of age. Mercaptoethanol-susceptible agglutinin (○); mercaptoethanol-resistant agglutinin (△).

chicken serum was taken 7 days after an injection of 2×10^{10} goat erythrocytes and separated on a sucrose density gradient. Warm agglutinin predominated in the rapidly sedimenting fractions, but cold agglutinin was present in both the fast and slowly sedimenting fractions (Fig. 2). The warm agglutinin in the fast sedimenting fractions (class I) was destroyed by ME, but the warm agglutinin (class II) in the slowly sedimenting fractions was ME-resistant (Fig. 3). A little of the cold agglutinin in the fast sedimenting fraction was ME-resistant (class I) but more of the cold agglutinin (class III) in the slowly sedimenting fraction was ME-resistant (Fig. 4).

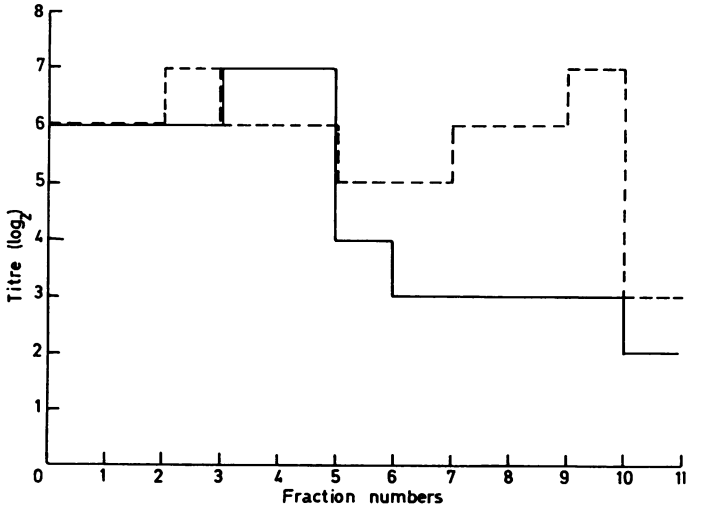

FIG. 2. Distribution of warm (—) and cold (- - -) agglutinins of immune adult sera in a sucrose density gradient.

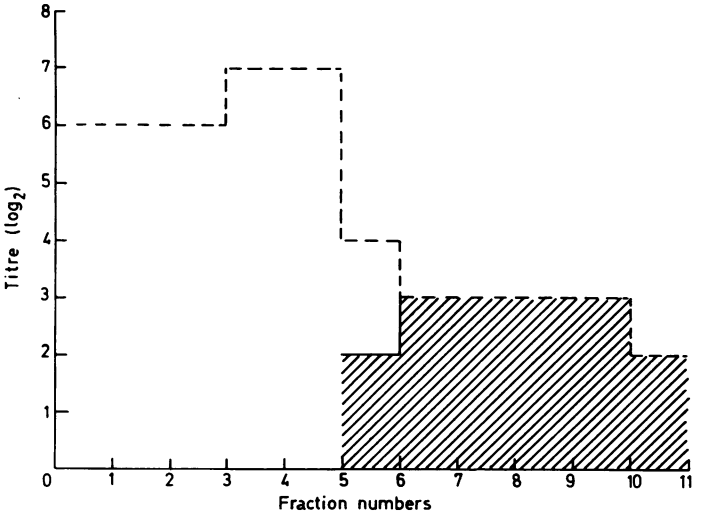

FIG. 3. Distribution of warm agglutinins of immune adult sera in a sucrose density gradient. Before (- - - -) and after (shaded) treatment of the fractions with 2-mercaptoethanol.

TABLE 1

CLASSIFICATION OF THE TYPES OF ANTIBODY TO GOAT ERYTHROCYTES BELIEVED TO HAVE BEEN DETECTED IN CHICKEN SERUM

Class (warm or cold)	Properties				
	ME		Heat (65° for 30 minutes)		Sedimentation rate
	Susceptible	Resistant	Labile	Stable	
I Warm	+	+	++	−	Fast (IgM)
Cold	++	−	++	−	Fast (IgM)
II Warm	−	++	+	+	Slow (IgG)
III Cold	+	+	−	++	Slow (IgA?)

One + indicates partial resistance or lability.

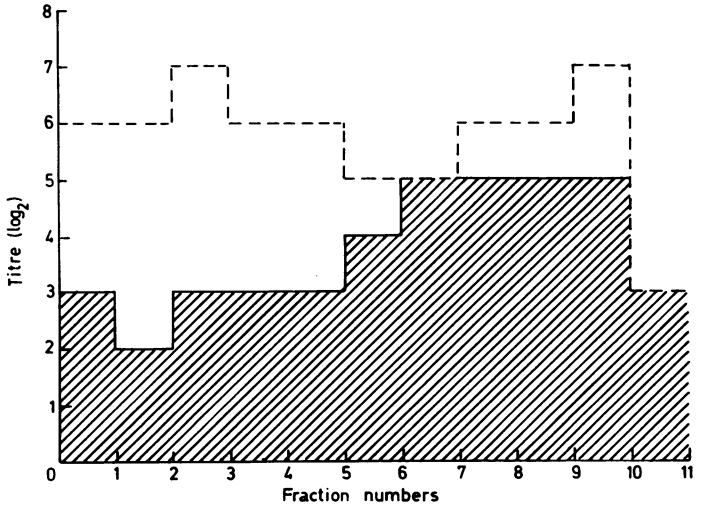

FIG. 4. Distribution of cold agglutinins of immune adult sera in a sucrose density gradient. Before (- - - -) and after (shaded) treatment of the fractions with 2-mercaptoethanol.

CLASS I AGGLUTININS

These agglutinins may be only one antibody which is capable of agglutinating at both 4° and at 37° but at different efficiencies. It is more probable that they are distinct warm and cold agglutinins with slightly differing susceptibilities to ME.

Heat treatment at 56° for 30 minutes is the standard procedure for removing complement in order that lysis may not interfere with agglutination. Unless otherwise stated, all sera have been so treated; it is probable that such treatment has partially destroyed some class I agglutinin, which is heat-labile.

Sera from sixty-four chickens ranging in age from hatching to 1-year-old were examined for natural antibody. Natural warm agglutinins were not detectable in serum previously heated at 56°, but natural cold agglutinin first appeared at 18 days of age (reciprocal titres 4–16). The titre of cold agglutinin in adult sera was similar. In sera which had not been heated at 56° only lysins could be detected earlier than the cold agglutinins. Those detected at very low titre (4) at hatching are probably antibody of maternal origin; higher titres (8) of natural lysins in sera from 7 to 13 days of age are probably natural antibody. All natural antibody was completely destroyed by ME and is probably class I antibody.

A single injection of a large number of goat erythrocytes in chicks from 6 to 15 days of age produced low titres of exclusively class I antibody (Table 2). Whereas 1-day-old chicks did not produce detectable agglutinins, both warm and cold agglutinin titres were greater in 22-day chick sera than in 15-day chick sera.

TABLE 2

AGGLUTININS PRODUCED AFTER A SINGLE INJECTION OF GOAT ERYTHROCYTES IN YOUNG CHICKS

Chicks		Dose of goat erythrocytes	Serum taken (days of age)	Reciprocal titres			
				37°		4°	
Age (days)	No.			Before ME	After ME	Before ME	After ME
1	8	3×10^9	8	<4	<4	—	—
6	8	1×10^9 to 2×10^{10}	13	7*	<4	—	—
8	6	1×10^8	15	4*	<4	9*	4*
9	4	1×10^{10}	15	22*	<4	21*	<4
15	5	2×10^8	22	28*	<4	64*	5*

* Geometric mean.

Double or treble injections of goat erythrocytes induced warm agglutinins at an earlier age than single injections. Chicks injected at 1 and 7 days of age produced class I warm agglutinin as early as 11 days of age.

Multiple injections of large doses of goat erythrocytes were given at 1, 3, 9 and 11 days after hatching in an attempt to induce hyperimmunity at an early age (Table 3). In eight out of nine chicks cold agglutinin titres (class I and perhaps some class III) were only slightly higher than those produced after a single injection into 9-day-old chicks, but titres of warm agglutinins were much lower. The appearance of high titres of class I agglutinin in one bird of this group was accompanied by class II and III agglutinins.

When 4·5-week-old chickens were injected with goat erythrocytes the titres of warm and cold agglutinins (Table 4) were much the same as those of chicks immunized at 15 days of age (Table 2) except that slightly more ME-resistant agglutinin was produced. Both warm

TABLE 3

ANTIBODY PRODUCED BY YOUNG CHICKS AFTER FOUR INJECTIONS OF ABOUT 5×10^9 GOAT ERYTHROCYTES

Chicks		Reciprocal titres at 17 days of age					
		37°			4°		
Sera	No.	Before ME	After ME	Heated at 65° for 30 minutes	Before ME	After ME	Heated at 65° for 30 minutes
Normal	9	<4	<4	—	4(1), <4(8)	<4	—
Immune	8	4*	<4	4(2), <4(6)	35*	<4	8, 4, <4(6)
	1	32	8	<4	128	64	32

Number of sera at a given titre shown in parentheses.

* Geometric mean.

and cold agglutinins were equally destroyed by ME (Table 4). The presence of heat-stable agglutinins was confirmed by heating pooled sera from the eleven immune chickens at 65°. After heat treatment, sera were divided into two portions: one was titrated immediately, the other treated with ME in the usual manner and then titrated. Most of the warm agglutinin (class I) was rapidly destroyed by heat (Fig. 5) but some remained even after heating at 65° for 1 hour, probably because some class II agglutinin (which appears to be more heat-stable) was present. Again, the considerable proportion of the cold agglutinin (Fig. 6) which was rapidly destroyed by heat during the first 10 minutes is probably class I.

TABLE 4

ANTIBODY PRODUCED 7 DAYS AFTER A SINGLE INJECTION OF 3×10^9 GOAT ERYTHROCYTES INTO 4·5-WEEK-OLD CHICKENS

Chicken		Reciprocal mean titres*			
		37°		4°	
Sera	No.	Before ME	After ME	Before ME	After ME
Normal	9	<4	<4	<4	<4
Immune	11	44(100%)	6(14%)	68(100%)	12(18%)

Per cent agglutinin activity remaining, shown in parentheses.

* <4 is taken as unity in calculating the geometric means.

Adult chickens were injected with a weight-adjusted dose of goat erythrocytes in order to compare their antibody response (Table 5) with that of the 4·5-week-old group (Table 4). Titres of adult sera were twice as high as those of the younger group; the amount destroyed by ME or heat in the two groups was similar (Table 5). The four most heat-stable sera were selected from the adult group, pooled, and the relative heat stability of the warm and cold agglutinins compared (Fig. 7). The destruction of warm agglutinin increased with the time of heating up to 40 minutes but was less rapid than the 5·5-week sera (Fig. 5). This is probably due to rapid destruction of class I agglutinin during the first 20 minutes of heat treatment, followed by slower destruction of the more heat-stable class II agglutinin. Only approximately half the cold agglutinin was also rapidly destroyed by heat (Fig. 7).

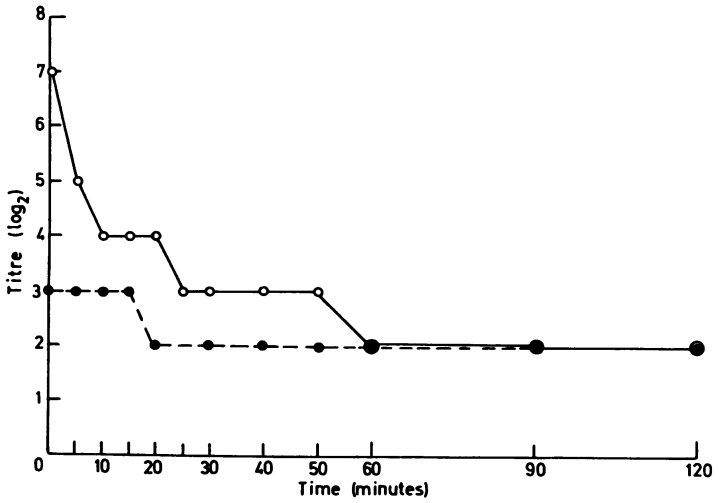

FIG. 5. Effect of heat treatment at 65° for 30 minutes (○) followed by treatment with 2-mercaptoethanol (●) on immune serum from 5·5-week-old chicken. Agglutinations performed at 37°.

TABLE 5

ANTIBODY PRODUCED BY ADULT CHICKEN IN RESPONSE TO A SINGLE DOSE OF 2×10^{10} GOAT ERYTHROCYTES

Chicken		Reciprocal mean titres			
		37°		4°	
Sera	No.	Before ME	After ME	Before ME	After ME
Normal	8	<4	<4	6	<4
Immune	9	94(100%)	19(20%)	174(100%)	44(25%)
Immune heated at 65° for 30 minutes		6(7%)		27(16%)	

Per cent agglutinin activity remaining, shown in parentheses.

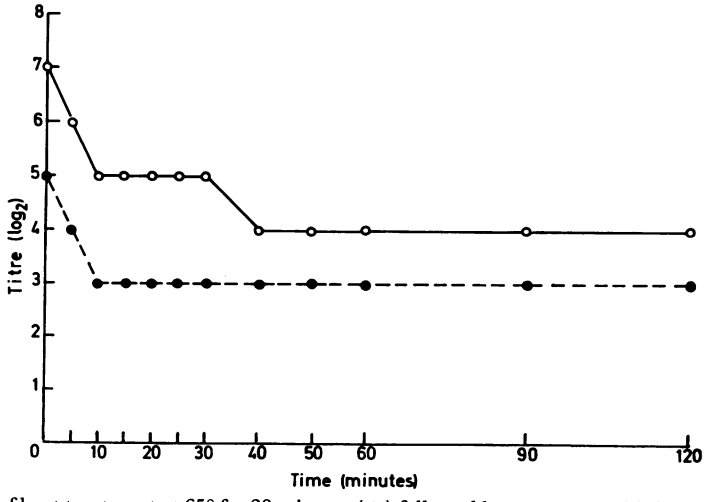

FIG. 6. Effect of heat treatment at 65° for 30 minutes (○) followed by treatment with 2-mercaptoethanol (●) on immune serum from 5·5-week-old chicken. Agglutinations performed at 4°.

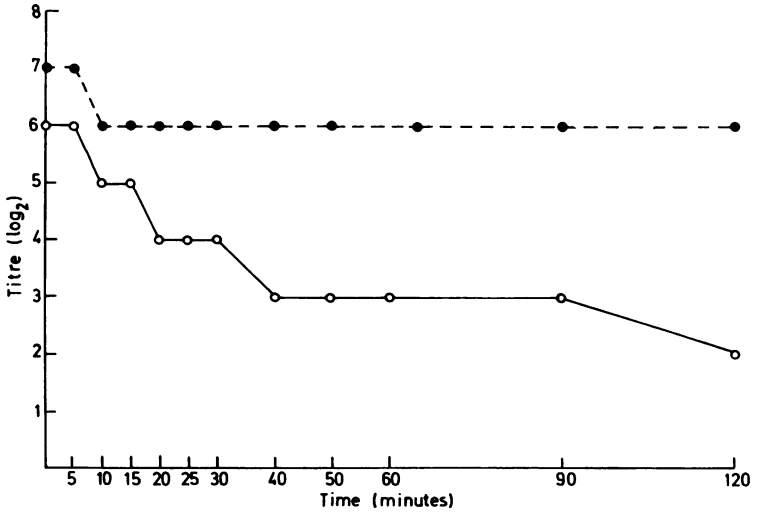

FIG. 7. Effect of heat treatment at 65° for 30 minutes on cold (●) and warm (○) agglutinins in selected adult immune sera.

CLASS II AGGLUTININ

Injections at 14 days of incubation, as well as at 5 and 11 days after hatching, did not elicit a greater response than a single injection of erythrocytes given at 9 days of age; but all the warm agglutinin at 17 days of age was ME-resistant. Class II agglutinin was detected in one of several chicks receiving multiple injections (Table 3). A little class II agglutinin was present in 5·5-week-old sera (Table 4) and probably accounts for the more heat-stable agglutinin remaining after the rapid destruction of class I agglutinin (Fig. 5). Although adult immune sera (Table 5) had higher titres, the class II agglutinin was the same percentage of the total as in the 5·5-week-old sera. A difficulty in testing whether young chicks can produce predominantly IgG (class II and III) antibody is that by the time the hyperimmunization schedule is completed the chicks are nearly immunologically

TABLE 6

PRODUCTION OF HYPERIMMUNE ANTIBODY BY SEVEN INJECTIONS OF GOAT ERYTHROCYTES (ABOUT 2×10^9) DURING 2–36 DAYS OF AGE

Chicken		Reciprocal mean titres			
		37°		4°	
Sera*	No.	Before ME	After ME	Before ME	After ME
Normal	4	<4	<4	16, 8, <4(2)	<4
Hyperimmune	5	169(100%)	84(50%)	169(100%)	84(50%)
Hyperimmune after heating at 65° for 15 minutes		49 (29%)		84(50%)	
Hyperimmune after heating at 65° for 30 minutes		11(7%)		42(25%)	

Per cent agglutinin activity remaining, shown in parentheses.

* Sera obtained 1 week after the last injection.

mature. Chicks were given seven injections of goat erythrocytes over a period from 2 to 36 days of age. The titres of warm and cold agglutinin were identical (Table 6) and higher than after a single injection of antigen. Only 50 per cent of the warm or cold agglutinin was destroyed after treatment with ME. Agglutinins showed similar heat stability to those produced after a single injection.

CLASS III AGGLUTININ

This agglutinin once appeared after multiple injections of erythrocytes were given to very young chicks (Table 3). It is present in 5·5-week-old chicken sera (Table 4). As ME was more destructive than heat to the cold agglutinin it is assumed that class III agglutinin is partially destroyed by ME (Fig. 6). This agglutinin was present in similar proportions in 5·5-week-old adult and hyperimmune sera.

DISCUSSION

As only small quantities of sera were obtainable from young chicks we have relied chiefly on ME to discriminate between IgM and IgG antibody. We were particularly influenced in this respect by the work of Benedict, Brown and Hersh (1963) who showed that treatment of anti-BSA chicken sera with 0·1 M ME for 30 minutes destroyed nearly all IgM antibody but only about 5 per cent IgG antibody. However, during the course of the present experiments we have detected two cold agglutinins (classes I and III), which are probably partially destroyed by ME, but do not appear to be IgM antibodies. IgG antibodies that are ME-susceptible have been reported in turtles responding to haemocyanin (Grey, 1963a), in frogs and goldfish responding to bacteriophage (Uhr, Finkelstein and Franklin, 1962), and in ducks (Grey, 1963b) and chickens (Rosenquist and Gilden, 1963) responding to BSA. Adler (1965) found that the first IgG antibody to appear in mice in response to sheep erythrocytes was partially destroyed by ME but the IgG antibody produced later in the response was ME-resistant.

Dissociated macroglobulin fragments may possess agglutinating activity which could account for our class I cold agglutinin appearing to have some ME-resistance. However, although antigen binding after dissociation of other macroglobulins has been reported (Jacot-Guillarmod and Isliker, 1964; Onoue, Yagi, Stelos and Pressman, 1964), ME-dissociated IgM anti-goat chicken antibody is no longer opsonic for goat erythrocytes (Solomon, 1966b).

Several macroglobulins which are heat-labile at 65° have been reported (Asherson and Dumonde, 1962; Mota, 1964; Adinolfi, 1965; Svehag, 1965). The heat stability of IgG antibody has been contrasted with the heat-lability of IgM antibody and the effect of heat compared with that of ethanethiol or ME (Murray, O'Connor and Gaon, 1965; Pike and Schulze, 1965). Immune antibody to bacteriophage in newborn rabbits is also heat-labile at 56°, but only because it is dependent upon complement for phage neutralization; in contrast, antibody from adults is independent of complement (Pernis, Ghezzi and Turri, 1963). In our work, we believe that the IgG antibody (class II) may not be very stable to heat at 65°, although more stable than IgM antibody.

The initial appearance of IgM antibody followed later by IgG antibody has frequently been shown to be the pattern of response in adult animals. Examples in young animals are the response of newborn guinea-pigs and foetal sheep to bacteriophage (Uhr, Finkelstein and Baumann, 1962; Silverstein, Uhr, Kraner and Lukes, 1963), of newborn rabbits to

S. paratyphi B vaccine (Bellanti, Eitzman and Smith, 1962), and of newborn infants to *Salmonella* vaccines (Smith and Eitzman, 1964).

Little is yet known about the relative affinity (avidity) of antibodies for antigen in the immune response of young animals. For example, in adult rabbits the development of the immune response proceeds via an IgM antibody of weak affinity to one of high affinity before IgG antibodies of high affinity appear (Svehag, 1965).

Říha and Svičulis (1964) reported that the primary response of adult chicken to sheep erythrocytes was predominantly IgM. Antibodies in the primary response of chicken to human serum albumin were both IgM and IgG, but antibody to *p*-azobenzoic acid hapten was exclusively IgM; further immunization induced IgG to the hapten. The anti-hapten antibodies were highly unstable (Říha, 1965). A single injection of BSA induced three distinct IgM antibodies and two separate IgG antibodies which were both ME-resistant (Benedict, Larson and Nik-Khah, 1963). However, only one of three IgG antibodies to BSA were found to be ME-resistant by Rosenquist and Gilden (1963).

The young chick rapidly becomes capable of making increased amounts of opsonizing antibody to erythrocytes during the first 2 weeks after hatching (Solomon and Tucker, 1963; Solomon, 1966a). The earliest detectable immune agglutinin (class I) in the present work appeared at 11 days of age. Bailey (1923) detected immune agglutinins to guinea-pig erythrocytes in 15-day chick sera. Both warm and cold class I antibodies were detectable by 15 days. Double or multiple injections of goat cells induced antibody formation earlier than single injections. One exceptional chick produced all three classes of antibody by 17 days after hatching. Older chicken (4·5 weeks) all produced the three classes of antibody.

Other workers studying the maturation of the immune response during development of the chicken have used bovine serum (Wolfe and Dilkes, 1948), bovine serum albumin (Wolfe, Mueller, Neess and Tempelis, 1957) and *Salmonella pullorum* (Buxton, 1954) as antigens. They found that the highest titres of antibody were often not obtained until from 14 to 20 weeks of age. Some quantitative maturation of the primary response to goat erythrocytes may occur from 5 to 24 weeks of age, but there was no change in the proportions of the three classes of antibody. It is possible to establish hyperimmunity to goat erythrocytes at 6 weeks of age when the proportion of class II antibody is considerably increased; but that of class III antibody remains the same as in the primary response.

When titres of IgM antibody have been high enough, then IgG antibodies have nearly always been detected. The haemagglutination method discriminates against the detection of IgG antibody because of the much greater agglutinating ability of IgM antibody. This leads us to the tentative conclusion that, on a molecular basis, the predominant type of antibody may be IgG even in the primary responses of young chicks, provided that large doses of erythrocytes are given.

ACKNOWLEDGMENTS

We wish to thank Professor A. Haddow, F.R.S., for his encouragement and support. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council and the British Empire Cancer Campaign for Research, and by the Public Health Service Research Grant No. CA-03188-08 from the National Cancer Institute, U.S. Public Health Service.

REFERENCES

ADINOLFI, M. (1965). 'Some serological characteristics of normal incomplete cold antibody.' *Immunology*, **9**, 31.

ADLER, F. L. (1965). 'Studies on mouse antibodies. II. Mercaptoethanol-sensitive 7S antibodies in mouse antisera to protein antigens.' *J. Immunol.*, **95**, 39.

ASHERSON, G. L. and DUMONDE, D. C. (1962). 'Characterisation of auto-antibodies produced in the rabbit by the injection of rat liver.' *Brit. J. exp. Path.*, **43**, 12.

BAILEY, C. E. (1923). 'A study of normal and immune hemagglutinins of the domestic fowl with respect to their origin, specificity and identity.' *Amer. J. Hyg.*, **3**, 370.

BAUER, D. C. and STAVITSKY, A. B. (1961). 'On the different molecular forms of antibody synthesised by rabbits during the early response to a single injection of protein and cellular antigens.' *Proc. nat. Acad. Sci. (Wash.)*, **47**, 1667.

BELLANTI, J. A., EITZMAN, D. V. and SMITH, R. T. (1962). 'Sequence of antibody component appearance in newborn rabbits.' *Fed. Proc.*, **21**, 30.

BENEDICT, A. A., BROWN, R. J. and HERSH, R. (1963). 'Inactivation of high and low molecular weight chicken antibodies by mercaptoethanol.' *Proc. Soc. exp. Biol. (N.Y.)*, **113**, 136.

BENEDICT, A. A., LARSON, C. and NIK-KHAH, H. (1963). 'Synthesis of chicken antibodies of high and low molecular weight.' *Science*, **139**, 1302.

BUXTON, A. (1954). 'Antibody production in avian embryos and young chicks.' *J. gen. Microbiol.*, **10**, 398.

GREY, H. M. (1963a). 'Phylogeny of the immune response. Studies on some physical, chemical and serological characteristics of antibody produced in the turtle.' *J. Immunol.*, **91**, 819.

GREY, H. M. (1963b). 'Production of mercaptoethanol sensitive, slowly sedimenting antibody in the duck.' *Proc. Soc. exp. Biol. (N.Y.)*, **113**, 963.

JACOT-GUILLARMOD, H. and ISLIKER, H. (1964). 'Scisson réberable des isoagglutinines 19S étude de fixation des subunités.' *Vox Sang. (Basel)*, **9**, 31.

KARTHIGASU, K., JENKIN, C. R. and TURNER, K. J. (1964). 'Nature of the opsonins in adult hen serum and developing chick embryos to certain gram-negative bacteria.' *Aust. J. exp. Biol. med. Sci.*, **42**, 499.

MOTA, I. (1964). 'The mechanism of anaphylaxis. I. Production and biological properties of "mast cell sensitizing" antibody.' *Immunology*, **7**, 681.

MURRAY, E. S., O'CONNOR, J. M. and GAON, J. A. (1965). 'Differentiation of 19S and 7S complement fixing antibody in primary versus recrudescent typhus by either ethanethiol or heat.' *Proc. Soc. exp. Biol. (N.Y.)*, **119**, 291.

NOSSAL, G. J. V., ADA, G. L. and AUSTIN, C. M. (1964). 'Antigens in immunity. II. Immunogenic properties of flagella, polymerised flagellin and flagellin in the primary response.' *Aust. J. exp. Biol. med. Sci.*, **42**, 283.

ONOUE, K., YAGI, Y., STELOS, P. and PRESSMAN, D. (1964). 'Antigen binding activity of 6S subunits of β_2-macroglobulin antibody.' *Science*, **146**, 404.

PERNIS, B., GHEZZI, I. and TURRI, M. (1963). 'Properties of phage-neutralising antibodies produced by newborn rabbits.' *Nature (Lond.)*, **197**, 807.

PIKE, R. M. and SCHULZE, M. L. (1965). 'The relative heat stability of antibodies in chromatographic fractions of rabbit antisera to various antigens.' *J. Immunol.*, **94**, 31.

ŘÍHA, I. (1962). 'Antibody formation in young rabbits immunized within the first days after birth.' *Symposium on the Mechanisms of Immunological Tolerance* (Ed. by M. Hašek, A. Lengerová and M. Vojtišková), pp. 103–6. Publishing House of Czechoslovak Academy of Sciences, Prague.

ŘÍHA, I. (1965). 'The formation of specific 7S and macroglobulin type antibodies in chickens.' *Symposium on the Molecular and Cellular Basis of Antibody Formation* (Ed. by J. Šterzl), pp. 253–9. Publishing House of Czechoslovak Academy of Sciences, Prague.

ŘÍHA, I. and SVIČULIS, A. (1964). 'Antibodies against hapten of 7S and macroglobulin type in chickens.' *Folia microbiol. (Praha)*, **9**, 45.

ROSENQUIST, G. L. and GILDEN, R. V. (1963). 'Chicken antibodies to bovine serum albumin. Molecular size and sensitivity to 2-mercaptoethanol.' *Biochim. biophys. Acta (Amst.)*, **78**, 543.

SILVERSTEIN, A. M., UHR, J. W., KRANER, K. L. and LUKES, R. J. (1963). 'Fetal response to antigenic stimulus. II. Antibody production by the fetal lamb.' *J. exp. Med.*, **117**, 799.

SMITH, R. T. (1960). 'Response to active immunisation of human infants during the neonatal period.' *Ciba Symposium on Cellular Aspects of Immunity* (Ed. by G. E. W. Wolstenholme and M. O'Connor), p. 348. Churchill, London.

SMITH, R. T. and EITZMAN, D. V. (1964). 'The development of the immune response.' *Pediatrics*, **33**, 163.

SOLOMON, J. B. (1966a). 'Induction of antibody formation to goat erythrocytes in the developing chick embryo and effects of maternal antibody.' *Immunology*, **11**, 89.

SOLOMON, J. B. (1966b). 'The appearance and nature of opsonins for goat erythrocytes during the development of the chicken.' *Immunology*, **11**, 79.

SOLOMON, J. B. and TUCKER, D. F. (1963). 'Ontogenesis of immunity to erythrocyte antigens in the chick.' *Immunology*, **6**, 592.

SVEHAG, S.-E. (1965). 'The formation and properties of polio-virus neutralising antibody. V. Changes in the quality of 19S and 7S rabbit antibody.' *Acta path. microbiol. scand.*, **64**, 103.

UHR, J. W. (1964). 'Heterogeneity of the immune response.' *Science*, **145**, 457.

UHR, J. W., FINKELSTEIN, M. S. and BAUMANN, J. B. (1962). 'Antibody formation. III. The primary and secondary response to bacteriophage ΦX174 in guinea-pigs.' *J. exp. Med.*, **115**, 655.

UHR, J. W., FINKELSTEIN, M. S. and FRANKLIN, E. C. (1962). 'Antibody response to bacteriophage ΦX174 in non-mammalian vertebrates.' *Proc. Soc. exp. Biol. (N.Y.)*, **111**, 13.

WOLFE, H. R. and DILKES, E. (1948). 'Precipitin production in chickens. III. The variation of antibody response as correlated with the age of the animal.' *J. Immunol.*, **58**, 245.

WOLFE, H. R., MUELLER, A., NEES, J. and TEMPELIS, C. (1957). 'Precipitin production in chickens. XVI. The relationship of age to antibody production.' *J. Immunol.*, **79**, 142.