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Fowl Antibody

VIII. A COMPARISON OF NATURAL, PRIMARY AND SECONDARY ANTIBODIES TO ERYTHROCYTES IN HEN SERA; THEIR TRANSMISSION TO YOLK AND CHICK

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Summary. Despite some quantitative differences, the 'natural' antibody to erythrocytes present in normal fowl serum was found to be transmitted to yolk and chick in the same manner as induced antibody to sheep red cells. Estimations of direct and indirect agglutination titres and of haemolytic activity in the sera and yolks of hens after one and two injections of sheep red cells showed differences between the antibodies of sera and yolks, and also between those produced during the primary and secondary responses. The proportion of non-agglutinating antibodies was higher, and the haemolytic activity lower, after the second injection. Antibodies transferred to yolk and chick were mainly non-agglutinating and not haemolytic.

INTRODUCTION

Normal adult fowl serum contains antibody against the erythrocytes of all species so far tested, including avian ones. These antibodies first appear between 3 and 5 weeks of age. Antibodies to rabbit, rat, duck and pigeon red cells are found earlier (by about 10 days) than anti-sheep, guinea-pig, human, mouse or turkey red cell antibodies (Bailey, 1923; Janković and Isaković, 1960). Adult levels are reached between 6 and 25 weeks of age. Titres vary with the breed of bird and in different laboratories, but depend mainly on the species of cells used, being highest (of the order 1:200) against rabbit, rat, pig and mouse cells, and lowest (1:10) against goat, horse and guinea-pig cells.

The transmission of antibody to the chick via the yolk has been demonstrated with toxins, viruses, bovine serum albumin (BSA) and bacterial antigens (Ramon, 1928; Buxton, 1952; Patterson, Youngner, Weigle and Dixon, 1962), as has the presence of γ-globulin in yolk (Williams, 1962) and its appearance in embryo serum on the 14th day of incubation (Kaminski and Durieux, 1956). A recent study has shown different modes of transmission for the natural and induced opsonins of hen serum against certain Gram-negative bacteria (Karthigasu, Jenkin and Turner, 1964). The presence of passively acquired antibody might well account for the failure of most attempts (Smith, 1961) to induce tolerance in the chick to any erythrocytes other than fowl, and the small degree of unresponsiveness found by Simonsen (1956) with human red cells.

This study was undertaken at first to see whether passively acquired natural antibodies to red cells could be detected in very young chicks and in yolks in the same way as induced

antibody to sheep red cells (SRC). Results obtained by direct agglutination showed that immune anti-SRC antibodies produced after a single injection were transmitted to a much lesser extent than antibody to BSA, influenza and Newcastle disease viruses, where serum and yolk levels had been found almost the same (Patterson *et al.*, 1962; Barhouma, 1963). The tests were therefore repeated after a second injection to see whether the first result was due to the nature of the antigen or of the primary response, and also in the hope of clarifying the differences found between primary and secondary antibody (Dreesman, Larsen, Pinkard, Groyon and Benedict, 1965).

MATERIALS AND METHODS

Sera and yolks

Sera were obtained from twenty 8-month-old hens (Hybrid 505 × White Leghorn) in full lay. The anti-sheep red cell (SRC) agglutination titres were measured both before and after heating the serum to 56° for 30 minutes. Heating abolished the lysis that occurred with almost all the sera to dilutions of 1:8 or more, but did not change the agglutinin titres. These ranged from 1:8 to 1:128. Six birds with the highest titres were kept for studies of naturally occurring antibody in their sera and yolks.

The fourteen remaining hens were injected intravenously with 1 ml of a 20 per cent suspension of washed SRC, and were bled 7 and 14 days later. Eggs were collected daily for 3 weeks. The eggs were fertile and a number laid 10–15 days after injection were incubated and hatched.

Fifteen weeks after the first injection the twelve surviving hens were again injected with 1 ml of 20 per cent SRC and bled, 7, 14, 23 and 41 days later. The eggs were collected daily.

Yolk extracts

The yolks were separated from the whites, and placed in a Petri dish. The contents were mixed with a syringe, fitted with a wide bore needle (13 G). Two millilitres of yolk were thoroughly mixed with 8 ml of saline, and centrifuged at 3000 rev/min for 15 minutes. The supernatant was stored at −20°, and used without further treatment.

Preliminary tests showed that dilutions of yolk below about 1:160 inhibited lysis by fowl complement; this effect disappeared after heating at 56° for 30 minutes. Yolk extracts used for complement fixation tests were therefore heated.

Normal chick sera

Sera were obtained from one large batch of chicks by killing or by bleeding on successive days. The chicks were of the same breed as those used before but were derived from younger hens. For the first 7 days it was necessary to pool blood from two chicks.

Sheep, goat and rabbit red cells

Cells were obtained from the same animals throughout, and kept in Alsever's solution for not less than 3 days and not more than 2 weeks. They were washed in saline three times before use.

The '10 per cent' suspensions were standardized spectrophotometrically after lysing an aliquot in 0·1 per cent Na_2CO_3 solution (Kabat and Mayer, 1961).

Rabbit anti-fowl globulin serum

Serum was prepared by giving a rabbit two injections of a washed specific precipitate made with fowl anti-BSA serum, in Freund's adjuvant. This antiserum gave a single band on immunoelectrophoresis against undiluted fowl serum, and was used unheated and unabsorbed for all antiglobulin tests.

Diluents

Unless otherwise stated, 0·9 per cent NaCl buffered with phosphate at pH 7·2 was used. In complement fixation tests veronal buffered saline (VBS) with added Ca^{++} and Mg^{++} was used (Kabat and Mayer, 1961).

For agglutination tests involving rabbit red cells (RRC), which sometimes do not give clear negative controls in saline, all dilutions and cell suspensions were made in a 3·3 per cent solution of $MgSO_4 \cdot 7H_2O$ (Joysey, 1955). Parallel titrations in saline showed no discrepancies.

Direct haemagglutination

Doubling dilutions of serum or yolk extract were made in 0·25-ml volumes of saline. Initial dilutions (taking into account the 1:5 dilutions of the yolk extract) were generally 1:20 for the immune systems. An equal volume of a 0·25 per cent red cell suspension was added. The tubes were shaken and placed in a water bath at 37° for 1 hour. Readings were made after the cells had settled and again on the following day.

Indirect haemagglutination

Mixtures of red cell suspension and antiserum or yolk extract dilutions, usually starting at 1:400, were incubated at 37° for 30 minutes. The tubes were centrifuged at 1500 rev/min for 5 minutes. The supernatants were removed and the sedimented cells resuspended in 0·25 ml saline. Tubes showing agglutination at this stage were discarded, and recorded as showing 'direct agglutination'. The remaining cell suspensions were washed twice with 0·75 ml of saline. The cells were finally resuspended in 0·5 ml of a 1:400 dilution of rabbit anti-fowl globulin serum and re-incubated for 1 hour.

Haemolytic complement (C′)

Two batches of fresh pooled normal serum from 5- to 6-month-old birds were used. The removal of the natural haemolysin by absorption in the cold (Rose and Orlans, 1962) proved to be difficult and was, in fact, never completely successful. Absorption with goat red cells (GRC) was used because Bailey (1923) had found that GRC, while not lysed or agglutinated by normal fowl serum, could remove all the normal antibody to SRC. Some lysis did occur unless the system was carefully kept below 4°, but the GRC were indeed found as effective as SRC in absorbing the lysins. However, even repeated absorptions with goat or sheep cells and combinations of the two, using equal volumes of packed cells and of serum, gave C′ preparations that lysed unsensitized SRC to a dilution of 1:16. In contrast to this a single absorption at 37° with ½ volume of SRC sufficed to remove all agglutinins from heated normal serum (see Table 6).

The activity of the twice absorbed C′ preparation, used to compare the sensitizing properties of various antibodies, with a standard sensitizing serum, is shown in Table 1.

TABLE 1

THE LYSIS OF SRC SENSITIZED WITH DILUTIONS OF AN ANTI-SRC FOWL SERUM, IN THE PRESENCE OF VARYING LEVELS OF FRESH FOWL SERUM ABSORBED TWICE WITH GOAT AND SHEEP CELLS RESPECTIVELY

Dilutions of twice absorbed complement	Dilution of sensitizing serum						
	80	160	320	640	1280	2560	S
1:45	4	4	4	4	(4)	0	0
1:60	4	4	4	C	0	0	0
1:75	4	4	1	0	0	0	0
1:90	4	3	C	0	0	0	0

Graded 4 = 100 per cent lysis, C = less than 25 per cent.

Haemolytic activity of the antibody

Doubling dilutions of the heated immune sera or yolk dilutions were made in 0·25-ml volumes of VBS, starting at 1:80. A 0·5 per cent suspension of SRC in VBS was added (0·25 ml), and the tubes thoroughly shaken.

Freshly diluted complement (0·25 ml) was added, to give a final dilution of 1:60. The contents of the tubes were mixed again, and incubated for 1 hour at 37° with shaking at 20-minute intervals. Unlysed cells were spun down and results recorded as 4, 3, 2, 1, C or 0, to represent 100, 75, 50, 25 per cent, trace or no lysis, respectively.

Sera obtained 7 days after the first and second injections were all titrated at the same time to avoid discrepancies due to deterioration of the C′ material.

Some of the results are tabulated as $\log_2$ of the reciprocal of the end point dilution. Therefore, a difference of 1, represents a difference of one tube in a series of doubling dilutions. Some examples are given in the table below.

1/dilution	4	8	60	160	640	3200	5120	25,000	100,000	400,000
$\log_2$	2	3	5·906	7·322	9·322	11·644	12·322	14·644	16·609	18·609

RESULTS

INDUCED ANTIBODY

Seven days after the first injection of SRC thirteen out of fourteen hens had high levels (above 1:2560) of agglutinating antibody in their sera (Table 2). These had declined by an average of two tubes (or doubling dilutions) 7 days later. Yolk antibody was measured in 3–4 eggs per hen in eggs laid between 9 and 15 days after injection. Most of the yolks had already reached peak titres on day 9, all had by day 12 (Table 2) and there was little decline by day 15. An unexpected finding was that the direct agglutination titres of the yolk extracts were consistently much lower (none above 1:320) than those of the corresponding sera, the mean difference being 4·6 tubes (Table 3). In the sera of chicks hatched from eggs laid during the same periods, agglutination titres were 1:20 or less (Table 2).

Seven days after the second injection the direct serum agglutination titres rose to almost the same mean value as after the first injection. Their decline was, however, very much slower: a mean decrease of two tubes 6 weeks after injection compared with 2 weeks in the primary response. Yolk titres reached their peak 2–5 days later than serum titres and

TABLE 2

AGGLUTINATION AND HAEMOLYTIC TITRES AGAINST SRC OF SERA AND YOLK EXTRACTS OBTAINED IN THE COURSE OF THE PRIMARY AND SECONDARY RESPONSES

		Agglutination (log_2)									Haemolysis (log_2)	
		Primary response			1-day-old chicks from eggs of	Secondary response					Primary response	Secondary response
Days after injection:		7	12	14	16–18	7	11–12	14	23	41	7	7
Hen No.		Serum	Yolks	Serum	Serum	Serum	Yolks	Serum	Serum	Serum	Serum	Serum
961	Direct	11·6	7·3	9·3	4·3	11·6	8·3	9·3	8·8	8·3	11·3	9·3
	Indirect	16·6	15·6	—	14·6	17·2	16·6	—	15·6	—		
966	Direct	12·6	8·8	11·3	—	11·6	8·3	11·3	10·3	10·3	> 12·3	10·3
	Indirect	17·6	16·3	—		17·6	17·6	—	16·6	—		
968	Direct	12·3	7·8	11·3	4·3	12·3	8·3	11·3	10·8	10·3	11·3	11·3
	Indirect	17·6	16·0	—	13·6	17·6	17·0	—	16·6	—		
969	Direct	11·6	7·3	9·3	—	10·6	7·8	8·8	8·3	8·3	10·3	8·3
	Indirect	14·6	14·6	—		16·6	16·6	—	15·6	—		
970	Direct	11·6	5·3	9·3	—	10·3	8·3	9·3	9·3	9·3	10·3	8·3
	Indirect	15·6	12·6	—		16·6	16·6	—	15·6	—		
973	Direct	11·3	7·8	9·3	< 4·3	10·6	6·8	8·3	8·3	7·8	11·3	8·3
	Indirect	17·2	16·3	—	14·6	16·6	15·6	—	15·6	—		
974	Direct	12·6	7·8	11·3	—	12·3	8·6	12·3	11·3	11·3	12·3	12·3
	Indirect	16·6	16·3	—		17·6	17·6	—	16·6	—		
975	Direct	12·6	7·3	8·3	—	10·6	8·3	9·3	8·3	8·3	11·3	9·3
	Indirect	16·6	15·6	—		17·2	17·6	—	14·6	—		
976	Direct	8·6	2·3	7·3	—	12·6	7·3	11·3	10·3	9·8	8·3	12·3
	Indirect	11·2	10·6	—		15·6	16·6	—	13·6	—		
978	Direct	11·6	7·3	9·3	—	11·6	—	10·3	9·3	9·3	11·3	10·3
	Indirect	15·6	—	—		17·6	—	—	14·6	—		
979	Direct	12·6	8·3	10·3	—	12·6	9·3*	11·3	11·3	10·3	> 12·3	11·3
	Indirect	17·2	—	—		18·2	—	—	16·6	—		
980	Direct	12·6	8·6	10·3	4·3	12·3	7·8	11·3	11·3	9·3	12·3	12·3
	Indirect	16·6	15·6	—	14·6	17·6	17·2	—	14·6	—		
Mean	Direct	11·8	7·2	9·7	4·3	11·6	8·1	10·3	9·8	9·4	11·2	10·3
	Indirect	16·1	15·0	—	14·4	17·2	16·9	—	15·5	—		

* First egg, laid 15 days after injection.

showed only a one tube decline 4 weeks after injection. As in the primary response, yolk agglutination titres were never above 1 : 320.

Indirect agglutination results, and particularly the differences between direct and indirect readings (Table 3), demonstrate some differences between the primary and secondary responses and also between serum and yolk antibody:

(a) The proportion of non-agglutinating (incomplete) antibody was greater in the secondary than in the primary response.

(b) The proportion of incomplete to agglutinating antibody was much greater in yolks (and in chick sera) than in the immune hen sera. The largest difference between the two was ten tubes for the chick sera, followed by eight and nine tubes for primary and secondary yolks, and four and six tubes for primary and secondary immune sera.

(c) The levels of incomplete antibody in yolks were only slightly lower than in the corresponding sera, one tube on average. If one equates undiluted serum with undiluted

TABLE 3

DIFFERENCES FOUND DURING PRIMARY AND SECONDARY RESPONSES BETWEEN (a) INDIRECT AND DIRECT AGGLUTINATION TITRES IN HEN SERA AND THE CORRESPONDING YOLKS, AND (b) PEAK LEVELS OF AGGLUTINATING AND INCOMPLETE ANTIBODIES IN SERA AND YOLKS OF THE SAME HEN

Hen No.	Primary response				Secondary response			
	Difference between agglutination titres (log_2) (indirect minus direct)		Difference between maximum serum titre and maximum yolk titre (log_2) (serum minus yolk)		Difference between agglutination titres (log_2) (indirect minus direct)		Difference between maximum serum titre and maximum yolk titre (log_2) (serum minus yolk)	
	Serum	Yolk	Direct	Indirect	Serum	Yolk	Direct	Indirect
961	5·0	8·3	4·3	1·0	5·6	8·3	3·3	0·6
966	5·0	7·5	3·8	1·3	6·0	9·3	3·3	0
968	5·3	8·2	4·5	1·6	5·3	8·7	4·0	1·6
969	3·0	7·3	4·3	0	6·0	8·8	2·8	0
970	4·0	7·3	6·3	3·0	6·3	8·3	2·0	0
973	5·9	8·5	3·5	0·9	6·0	8·8	3·8	1·0
974	4·0	8·5	4·8	0·3	5·3	9·0	3·7	0
975	4·0	8·3	5·3	1·0	6·6	9·3	2·3	−0·4
976	2·6	8·3	6·3	0·6	3·0	9·3	5·3	−1·0
978	4·0	—	4·3	—	6·0	—	—	—
979	4·6	—	4·3	—	5·6	—	3·3	—
980	4·0	7·0	4·0	1·0	5·3	9·4	4·0	0·4
Mean	4·3	7·9	4·6	1·1	5·8	8·9	3·3	0·2

yolk, the transfer of incomplete antibody appears quantitative and does not differ significantly in the primary and secondary responses.

(d) The ratio of agglutinating to incomplete antibody was the same 7 and 23 days after the second injection, indicating that the two types of antibody declined at the same rate.

The haemolytic activity of primary and secondary antisera is shown in the last two columns of Table 2. The difference between the two was rather greater than appears from the titres, since the primary antisera lysed more rapidly and completely than the secondary antisera, where lysis tended to be incomplete in the last two tubes.

With the more haemolytic primary antisera the haemolytic end points were almost the same as the direct agglutination titres. The titres of the secondary antisera were lower on average by 1·3 tubes. The non-agglutinating antibodies bore no relation to haemolysins. This was confirmed by the haemolytic tests with yolk extracts (results not shown) in which extracts containing large amounts of antibody (1:320 direct and 100,000 indirect) gave a partial lysis end point of 1:320 ($log_2 = 8·3$) with C′ at 1:60. Because of the residual haemolysin in the complement, it was not certain that the yolk antibody was in fact mediating lysis.

NATURALLY OCCURRING ANTIBODY

Anti-sheep and anti-rabbit red-cell agglutination titres of sera and yolks of normal hens are shown in Table 4. No direct anti-SRC agglutinins were found in yolks, but one extract agglutinated RRC to a dilution of 1:20. Antibody to SRC in yolk could be demonstrated by the antiglobulin method but the levels were lower than in the corresponding sera.

Agglutinin levels in the sera of young chicks are summarized in Table 5, and show that

TABLE 4

DIRECT AND INDIRECT ANTI-SRC, AND DIRECT ANTI-RRC AGGLUTINATION TITRES OF SERA AND YOLKS OF NORMAL ADULT HENS

Hen No.	Serum			Yolk		
	Anti-SRC direct	Anti-SRC indirect	Anti-RRC direct	Anti-SRC direct	Anti-SRC indirect	Anti-RRC direct
962	64	800	640	< 5	< 200	< 10
963	32	1600	640	< 5	30	< 10
964	64	800	640	< 5	200	< 10
965	64	400	1280	< 5	200	< 10
967	32	800	1280	< 5	30	20
972	32	200	320	< 5	10	Not done

up to 9 days after hatching most chicks possessed antibody to rabbit and/or sheep cells, the latter only demonstrated by the antiglobulin test. No antibody was detected between day 10 and 17; by day 21 the anti-RRC had risen to quite high levels, but indirect anti-SRC was not yet detectable. Despite the individual variations apparent in the table, it is clear that the natural antibody of normal fowl serum to erythrocytes was transmitted via the yolk to the young chick. It declined like any other passive antibody before the appearance, at about 3 weeks, of newly synthesized antibody which, unlike the passive antibody, was haemolytic.

The use of RRC instead of SRC for the detection of low levels of antibody in normal yolks and chick sera was prompted by the higher titres obtained with these cells and normal adult fowl sera (Table 4). These higher titres and also the early appearance of anti-RRC in chicks (Janković and Isaković, 1960) were thought to be due to the greater 'agglutinability' of these cells resulting in higher sensitivity of agglutination tests.

Some of the chicks (4 and 7 days old) had antibody to SRC but not to RRC, while the reverse was true in some of the older chicks (Table 5). Cross-absorption with SRC and RRC of pooled heated serum from hens 962 and 965 gave the results in Table 6. Despite one and two tube reductions in the heterologous titres, the natural antibody had some specificity for each type of cell.

The presence of common antigen(s) on SRC and RRC was confirmed by the increases in anti-RRC titres (from 1:320 to at least 1:3200) in the sera of hens that had received a single injection of SRC.

TABLE 6

DIRECT AND INDIRECT AGGLUTINATION TITRES AGAINST SRC AND RRC OF HEATED POOLED HEN SERA (962 AND 965) AFTER ONE AND TWO ABSORPTIONS OF PACKED CELLS OF EACH TYPE

Titration with:	State of normal serum				
	Unabsorbed	Absorbed SRC	Absorbed SRC 2 ×	Absorbed RRC	Absorbed RRC 2 ×
SRC direct	128	< 16	< 16	32	32
SRC indirect	2000	16	< 16	256	256
RRC direct	4000	2000	2000	16	< 16
RRC indirect	4000	4000	4000	< 128	< 16

TABLE 5

ANTI-RRC AGGLUTINATION AND SOME INDIRECT ANTI-SRC TITRES OF NORMAL YOUNG CHICKS

Reciprocal of serum dilution		Age of chicks (days)																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	23	24	27	34
Direct anti-rabbit red cells	2	++	++	++	−	++	++	−	++	+	−	−	−	?	+	?	−	+	(+)	+	+	L	L	L	L	L
	4	++	++	++	−	(+)	++	−	++	(+)	−	−	−	−	−	−	−	?	−	?	−	+++	?	+++	++	++
	8	?	(+)	?	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+++	?	++	++	++
	16	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+++	−	+	++	++
	32	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	−	+	−	++
	64	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	±	−	(+)
	128	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	?
Indirect anti-sheep red cells		<20	30	10	20	30	5	5					<5	<5						<5	<10	<40				

DISCUSSION

There are few clear-cut criteria to distinguish between the primary and the secondary response in the fowl. None was found at the cellular level by White (1963) who studied the response in the spleen to human serum albumin. There is little difference in the speed or size of response after one or several injections unless the interval between injections is rather great, 5–10 weeks in adult fowls (Hofstad, 1953; Wolfe, Amin, Mueller and Aronson, 1960); the highest secondary response was found after a 36-week interval (Blazkovec and Wolfe, 1965).

One clear difference between the primary and secondary responses to BSA is a much greater persistence of antibody in the latter. The antibody found in large amounts 7 days after a single primary injection, is usually no longer detectable 3 weeks later. We have found precipitating antibody 1 year after the last of three spaced injections of BSA; Gilden and Rosenquist (1963a, b) have found continuing anti-BSA synthesis 470 days after the last of two injections given at an interval of 50 days. The slower rate of decline of secondary antibody has now been confirmed with the SRC system. The rapid return to normal levels of agglutinins and lysins after a single injection of SRC indicated that a primary type of response had occurred, despite the presence of natural antibody.

Despite some workers' failure to detect any 19S antibody (Rosenquist and Gilden, 1963; Orlans, Richards and Rose, 1964) there is now strong evidence that such antibody is produced, but that it constitutes only a small proportion of the primary anti-BSA measured by precipitation or antigen binding (Dreesman *et al.*, 1965); also that its production may depend on the nature of the antigen rather than on the duration of immunization (Szenberg, Lind and Clarke, 1965). The most striking difference between primary and secondary anti-BSA, found by Benedict, Hersh and Larson (1963) is that the primary 7S antibody (separated by sucrose gradient ultracentrifugation) does not agglutinate tanned SRC coated with BSA. This has been confirmed by us with BSA coated fowl red cells and 7S anti-BSA fractionated on Sephadex G-200. Secondary 7S anti-BSA does haemagglutinate, though less efficiently than the primary 19S material.

The differences between primary and secondary antibody and also the naturally occurring anti-SRC are as follows:

	Normal	Primary	Secondary
Direct agglutination	x	y	z
Indirect agglutination	$x+3{\cdot}6$	$y+4{\cdot}3$	$z+5{\cdot}8$
Haemolysis	x	y	$z-1{\cdot}3$
Transmission to yolk			
Agglutinating antibody	?	$y-4{\cdot}6$	$z-3{\cdot}3$
Non-agglutinating antibody	$x+0{\cdot}4$	$y+3{\cdot}2$	$x+5{\cdot}3$

Where $x = 5{\cdot}6$, $y = 11{\cdot}8$, $z = 11{\cdot}6$ and all values are $\log_2$, representing one doubling dilution. In relation to the directly agglutinating antibody, the proportion of incomplete antibody and also the degree of transmission to yolk increased with immunization; haemolytic efficiency, however, did not increase above normal levels after a primary injection, and declined after a second injection. This is further illustrated by hen 976 which failed to respond to the first injection (Table 2) and which, after the second injection, had the lower titres of incomplete and of yolk antibody (Table 3) associated with the primary response. The results are compatible with the presence of 19S antibody in the normal and primary sera, as only small amounts of this would account for the strong agglutinating

and lytic activities of the sera. However, the increased amount of incomplete antibody in the secondary response is at variance with the findings for the BSA system.

The nature of the antibody detected by the antiglobulin test is always open to question, since the absence of direct agglutination could be due to the nature of the red cell antigen and not of the antibody. In these experiments the presence of both agglutinating and incomplete antibodies, and their widely different proportions in the serum and yolk of the same hen, strongly suggest that they belong to different types of immunoglobulins. There is no doubt, because of the complexity of the red cell antigen, that antibodies of different specificities are present in these antisera; but there is no reason to suppose that any of these should be transmitted preferentially, unless they also belonged to different molecular species.

Ultracentrifugal analysis of specific precipitates redissolved in excess antigen has shown the presence of some antibody that combined with only one molecule of antigen (Orlans *et al.*, 1964). The existence of a precipitating (in high salt) anti-BSA that cannot haemagglutinate BSA coated red cells and the results with the anti-SRC system also suggest that fowl antisera may contain a proportion of univalent antibody.

Yolk antibody to SRC reached peak levels some 5 days later than serum antibody, as Patterson *et al.* (1962) found with antibody to BSA and influenza. When measured by the indirect method yolk titres were either equal to or slightly lower than serum titres and showed no sign of 'selective concentration of 7S γ_2-globulin' proposed by Malkinson (1965). Yolk antibody measured by precipitation (in 8 per cent NaCl) or by haemagglutination inhibition and neutralization in the case of virus antigens (Patterson *et al.*, 1962; Barhouma, 1963) was similar to serum levels, and these methods showed no qualitative differences between serum and yolk antibodies. Buxton (1952), however, using *Salmonella pullorum* as antigen found much lower levels of agglutinating antibody in yolks than in sera of immunized hens and a preponderance of non-agglutinating antibody (demonstrated with an anti-livetin serum) in the sera of young chicks and embryos after the 11th day of incubation.

The complement fixing properties of yolk antibody have not been studied before and our results show that if any haemolytic antibody is present at all, it is there in very small amounts compared with that found in serum.

The lack of species specificity of natural anti-erythrocyte antibody compared with the induced antibody, reported by Bailey (1923), was not found in our normal adult sera. The main differences between normal and induced antibody are consonant with a higher 19S content in the former. Karthigasu *et al.* (1964) found that opsonins for rough strains of bacteria in normal adult hen sera consisted of both 19 and 7S globulins; both types were present in the yolk sac, but only the 7S variety was transmitted to the chick before hatching. No such difference between yolk and newly hatched chick sera was detected by our methods, either with natural or induced antibodies. Whatever its molecular species, it is clear that enough of the natural antibody is transmitted to account for the immune clearance of labelled goat red cells by the 15-day embryo (Solomon, 1965) and for difficulties in inducing tolerance to heterologous erythrocytes.

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