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Immunological Unresponsiveness to Protein Antigens in Rabbits

II. THE NATURE OF THE SUBSEQUENT ANTIBODY RESPONSE

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Summary. Rabbits made immunologically unresponsive by neonatal administration of HSA, HGG or BSA were given a course of intravenous injections of the respective antigens, adsorbed on alum, after a lapse of 13–27 months since the last administration of antigen. 8/12 responded to HSA, 4/5 to HGG, 9/10 to BSA, as judged by immune elimination of antigen, but this was delayed in onset and slow compared with that in previously untreated rabbits. The antibody formed was small in quantity and usually failed to precipitate with antigen.

The sedimentation coefficients of ^{131}I-labelled antigens, in the presence of excess antibody, were measured by ultracentrifugation through a sucrose density gradient. These showed that only small complexes were formed in some of the non-precipitating antisera. In one instance the diffusion coefficient of the complex was also measured, by a technique based on diffusion through agar gel. The calculated molecular weight of the complex, 330,000 indicated the presence of only two combining sites on the antigen.

Combination of the anti-HSA sera with an HSA fragment was also measured. Whereas the amount of the fragment bound by ordinary hyperimmune anti-HSA sera was about one-fifth the HSA bound, the amounts bound by the test sera were relatively much less. Some non-precipitating sera failed to bind the fragment, although they bound HSA.

These findings indicate that following neonatally induced immunological unresponsiveness the capacity to respond to antigen returns piecemeal in respect of different parts of the antigenic mosaic, and that it may be severely restricted. The theoretical implications are discussed.

INTRODUCTION

In most, if not all, rabbits made specifically unresponsive to human serum albumin (HSA), human γ-globulin (HGG) or bovine serum albumin (BSA) by administration of these antigens at birth, the capacity to make antibody eventually returns, provided that the state of unresponsiveness is not maintained by repeated contact with the antigens (e.g. Smith and Bridges, 1958). Although in such animals the antibody response to other antigens is comparable to that of normal rabbits, the response to the antigen to which they were formerly unresponsive proved to be much more feeble than that of normal rabbits. Thus immune elimination of intravenously administered ^{131}I-labelled antigen occurred, but little or no precipitating antibody appeared in the serum, and even the antigen-

binding capacity (Farr, 1958) was much less than might have been expected after the course of antigen administration which was employed. It has also been reported elsewhere (Humphrey, 1964) that a small number of rabbits actually became once more specifically unresponsive when antigen administration was continued. A possible explanation might be that whereas normal rabbits respond to HSA, HGG or BSA by making antibodies against several aspects of the antigenic mosaic on these molecules, rabbits which have been rendered unresponsive recover the capacity to respond only piecemeal, in respect of one or a few parts of the antigenic mosaic at a time. Thus further administration of the specific antigen, at a time when only partial recovery of responsiveness had taken place, might result in stimulation of antibody formation against some sites on the antigen, but reinforcement of unresponsiveness against others. If this were true it might be expected that among a group of animals so treated there would be some in which the response to further antigenic stimulation would be confined to antibody against only one or two sites on the antigen. Such antibody would bind with the antigen but would not form a precipitate, since there would be too few binding sites to permit an adequate antigen–antibody lattice structure to be built up—a hypothesis advanced and substantiated by Berson and Yallow (1962) to explain the non-precipitating character of human antibodies against insulin. Although the antigens employed were not ideally suited for the purpose, and hapten conjugated antigens such as were used by Cinader and Dubert (1955) in their studies on tolerance might have served better, the following paper presents evidence to support the view that the return of responsiveness in previously unresponsive rabbits may indeed be confined to a very few sites on the antigen.

MATERIALS AND METHODS

Rabbits

These were included among those used in the experiments described in the preceding paper (Humphrey, 1964).

Antigens

The HSA, HGG and BSA used are also described in the preceding paper.

The HSA fragment, obtained by partial hydrolysis of HSA with spleen cathepsin, was kindly supplied by Dr. Claude Lapresle and its preparation and properties have been described by him (Lapresle and Webb, 1960). It had a molecular weight *ca.* 12,000, sedimentation coefficient about 1·1S, and contained one tyrosine group per molecule.

For measurement of antigen combining power the various materials were iodinated with ^{131}I or ^{125}I by the ICI method (McFarlane, 1958) at a level of 0·5–1·0 atoms per molecule, and their specific radioactivities were 150–400 μc./mg. In order to remove free iodide from the fragment after iodination, the material was passed through a column of Sephadex G25 (Pharmacia A-B, Uppsala). This treatment separated the labelled fragment cleanly, and avoided the necessity for dialysis or the use of ion exchange resin.

Measurement of Antigen-Combining Capacity

This was performed by the ammonium sulphate precipitation technique of Farr (1958) in the case of HSA, BSA and the HSA fragment, and by the use of goat anti-rabbit γ-globulin (e.g. Skom and Talmage, 1956) in the case of HGG. The anti-rabbit γ-globulin

used was selected from several goat or horse antisera on the grounds that it would precipitate rabbit γ-globulin completely, but in the presence of control rabbit sera caused less than 10 per cent precipitation of [^{131}I]HGG. Some other antisera, which apparently reacted only with rabbit γ-globulin on electrophoresis, precipitated much larger amounts of the HGG under these conditions, and it proved impossible to remove this cross-reactivity by repeated absorption with HGG firmly adsorbed onto bentonite particles.

Precipitating Antibody

Precipitating antibody was tested for qualitatively by double diffusion in agar gel, and was measured by the quantitative precipitation method. Mixtures of antigen and serum were stored for 48 hours at 4° before washing the precipitates.

Measurement of Sedimentation Coefficients of Antigen–Antibody Complexes

This was done by ultracentrifugation in a sucrose density gradient as described by Charlwood (1963). One ml. of the antiserum to be tested, whose antigen-combining power had previously been ascertained, was first centrifuged at 3500 ***g*** for 30 minutes and then mixed with 0·5 ml. of 0·1 M phosphate buffer, pH 7, containing ^{125}I-labelled marker proteins and a quantity of ^{131}I-labelled antigen small enough to ensure that the mixture would contain marked antibody excess. After standing for 2 hours at room temperature, approximately 1 ml. of the mixture was layered in a plastic centrifuge tube onto 11·5 ml. of 0·1 M phosphate buffer, in which a gradient from 30 to 10 per cent sucrose had been set up 24 hours earlier. The tubes were centrifuged at 25,000 rev./min. in the No. 40 head of a Spinco model L preparative ultracentrifuge for 15 hours at 10–12°. At the end of the run the fluid was withdrawn, by piercing the bottom of the tube, in sixteen to eighteen equal fractions. These, and any residue deposited on the bottom of the tube, were counted in a pulse height analyser for ^{125}I and ^{131}I radioactivity. The distribution of the antigen and of the radioactive markers was plotted against the incremental volume of fluid withdrawn from the tube. Haemocyanin from *Maia squinado* (S_{20w} of the main component = 23·4 at pH 7·0—unpublished measurement by Dr. P. A. Charlwood) human γ-macroglobulin (S_{20w} = 19), human or monkey γ-globulin (S_{20w} = 6·7), HSA and BSA (S_{20w} = 4·3) were used as markers; two markers with different sedimentation coefficients, chosen so as not to cross-react with the antibody under test, were added to each tube. When the sedimentation coefficients of the various markers (in the presence of normal rabbit serum) were plotted against the incremental volumes of fluid withdrawn, the points lay on straight lines whose slopes were remarkably constant from tube to tube and from experiment to experiment, although the absolute positions varied somewhat. Although the centrifugation was conducted at 10–12° the relative positions of different components would reflect their S_{20w} values and it was therefore possible to estimate these for the antigen–antibody complexes with fair accuracy from the position of the peaks of radioactivity associated with them.

Measurement of Diffusion Coefficient of Antigen–Antibody Complexes

An indirect method was used, since it was impracticable to separate the small amounts of non-precipitating antibody from all the other serum proteins, in sufficient quantity to permit direct measurement by the usual means of the diffusion coefficient of the complex with antigen. Allison and Humphrey (1960) showed that the rates of diffusion from a small

well through agar gels of radioactively labelled proteins can be estimated with considerable accuracy by measuring the radioactivity present in small uniform cylindrical samples punched out from the agar at various times and at various distances from the well. If diffusion takes place from thin pencil sources through a uniform layer, the concentration C at any point in the agar, distant r from the centre of the source is given by

$$C = \frac{M}{4\pi h\, Dt} \cdot e^{-r^2/4Dt} \qquad (1)$$

where M = quantity of material initially present in the source; h = depth of layer; t = time allowed for diffusion; D = diffusion coefficient.

In practice, in order to contain sufficient material, the well must have a finite diameter (3·5 mm. was used), and the punched out samples (1 mm. diameter) have a concentration gradient within them. The former consideration introduces some uncertainty about the zero time, although this can in principle be eliminated by taking measurements at more than one time interval. Since a plot of the logarithm to the base e of the radioactivity per sample against the square of the distance from the centre of the well gives a straight line, it is possible by taking multiple samples at a given time to draw the best straight line with reasonable accuracy. As shown below, the slope of the line is inversely proportional to the diffusion coefficient of the labelled material, and hence the diffusion coefficients of two materials, allowed to diffuse under identical circumstances in the same plate, can be readily compared. The argument is as follows:

Taking logarithms in expression (1), and differentiating with respect to r^2, we get

$$\frac{d}{dr^2}(\log_e C) = \text{slope at time } t = \frac{1}{4\,Dt} \qquad (2)$$

Hence for two materials with diffusion coefficients D_1 and D_2, the slopes of whose lines at time t are S_1 and S_2,

$$S_1/S_2 = D_2/D_1 \qquad (3)$$

By the use of expression (3), provided that the value of t is large, errors in zero time can be neglected. Furthermore it is not necessary to measure accurately the amounts of radioactive materials introduced into the wells at the start of the experiment.

A major assumption in using such a method to compare the diffusion coefficients of antigen alone and of antigen–antibody complex is that each diffuses equally freely through the agar. This clearly would not hold for large complexes, but should do so for those of the size under investigation. Thus Allison and Humphrey (1960) reported that even a 3 per cent agar gel did not prevent penetration of molecules with molecular weight $2{\cdot}7 \times 10^6$ although those with molecular weight $6{\cdot}6 \times 10^6$ were held back. In the present work the agar concentration was 1·2 per cent, which was the lowest possessing sufficient gel strength to allow the punch samples to be taken satisfactorily and the wells to be cut without splitting the agar.

The actual experiments were carried out at about 15°. Carefully levelled flat-bottomed petri dishes were filled to a depth of 4–5 mm. with agar in saline buffered at pH 7 and containing 0·1 per cent sodium azide. After 24 hours at room temperature, during which the gel dried slightly, two well-separated wells (diameter 3·5 mm.) were cut. One was carefully filled to the brim with ^{131}I-labelled HSA (specific activity *ca.* 350 μc./mg.) in normal rabbit serum and the other with radioactive HSA in the test serum, the ratio of

HSA to antibody being chosen to ensure antibody excess. Four or five punch samples were taken at various distances from the centres after about 19 and 40 hours. Although the dishes were enclosed in polythene bags to prevent excessive evaporation, the wells became empty within an hour of filling. However, since the purpose was to compare diffusion coefficients rather than to make absolute measurements, and since the mass flow of fluid into the agar took place equally from both wells, this factor was unimportant.

TABLE 1

EFFECT OF INJECTION OF ANTIGEN INTO RABBITS PREVIOUSLY MADE UNRESPONSIVE

A single intravenous injection of a solution of 15 mg. of the original antigen labelled with ^{131}I was followed 1 week later by six intravenous injections of 2·5 mg. of antigen adsorbed on aluminium hydroxide during the course of the next 2 weeks. Blood samples were taken 8 days after the last injection.

Antigen	*Rabbit No.*	*Time interval since last injection of antigen (months)*	*Presence or absence of immune elimination**	*Precipitating antibody (μg./ml.)†*	*Antigen binding capacity (μg./ml.)† in presence of antigen concentration:*		
					750 μg./ml.	80 μg./ml.	10 μg./ml.
HSA	36/58	16	Very delayed	0	47	12	1·8
	54/58	16	Absent	0	0	0	
	129/58	16	Delayed	0	19	0	
	131/58	16	Delayed	0	38	18	8
	16/59	16	Very delayed	0	0	0	
	17/59	16	1°	0	249	44	9
	19/59	16	Very delayed	0	27	12	1·4
	20/59	17	1°	72	138	52	10
	38/59	17	Absent	0	10	3	1
	40/59	17	1°	0	70	36	9·5
	41/59	17	Absent	0	10	0	
	42/59	17	Absent	0	0	0	0·07
BSA	57/58	13	Absent	0	0	0	
	58/58	13	1°	25	123	47	
	59/58	13	Very delayed	0	0	0	
	81/58	13	Very delayed	0	0	0	
	95/58	13	1°	23	106	43	
	97/58	15	Very delayed	0	0	0	
	99/58	15	Very delayed	0	39	3	
	100/58	15	1°	0	41	19	
	45/59	15	1°	0	64	33	
	46/59	15	Delayed	0	24	15	
					25 μg./ml.	1 μg./ml.	
HGG	113/58	16	Absent	0			
	115/58	16	Very delayed	0	0	0·03	
	118/58	16	1°	0	2·1	0·33	
	21/59	16	Delayed	0	0·9	0·32	
	23/59	27	1°	0	5·0	0·64	

* Whole body radioactivity was measured on alternate days for 4 weeks.
† Blood taken 4 weeks after administration of ^{131}I-labelled antigen.

RESULTS

The present investigation concerns twelve rabbits which had proved unresponsive on one or more occasions to HSA; ten to BSA; and five to HGG. The main points relating to their previous histories are tabulated in Table 1. Between 13 and 27 months after the last administration of antigen each rabbit received an intravenous injection of 15 mg. of ^{131}I-labelled antigen, to test for the presence or absence of immune elimination. This was followed 1 week later by six injections each of 2·5 mg. of the same unlabelled antigen

adsorbed on alum, administered during the course of 14 days. One week after the last injection the rabbits were bled for serological examination. The elimination of the initial dose of [^{131}I]antigen was followed throughout the 4 weeks by measurement of whole body radioactivity. As may be seen from Table 1, none of the rabbits gave a secondary response; some were still unresponsive, but most gave a primary type response which was usually very weak and markedly delayed. Normal rabbits given a similar course of injections of these antigens would be expected to give a good antibody response, with at least 100 μg. and commonly more than 1000 μg. of precipitating antibody per ml. of serum. Nevertheless sera of rabbits in this experiment rarely contained precipitating antibody, although most of those from animals showing immune elimination bound significant amounts of antigen under the conditions of testing. Certain of these sera were selected for examination of the effect of their contained antibodies on the behaviour of antigen in the ultracentrifuge.

TABLE 2

SEDIMENTATION COEFFICIENTS OF ANTIGEN–ANTIBODY COMPLEXES

Serum No.	*Antigen*	*Antigen concentration (μg./ml. serum)*	*Antigen-combining power of serum (μg./ml.)*	*Distribution of Ag–Ab complex in density gradient (S values are relative to the S_{20w} values of the markers)*
131/58	HSA	2·5	38	Single peak, 11·7S
135/58*	HSA	2·5	>40	98 per cent in deposit*
17/59	HSA	2·5	249	Single peak, 11·6S
20/59	HSA	2·5	138	95 per cent in deposit; trace at ~23S
40/59	HSA	2·5	70	90 per cent in deposit; trace at ~22S
100/58	BSA	2·7	41	Single peak, 11·2S
45/59	BSA	2·7	64	25 per cent in deposit; 70 per cent ~22S; 5 per cent ~10·5S
46/59	BSA	2·7	24	55 per cent in deposit; 9 per cent ~23S; 21 per cent ~17S; 15 per cent ~12·5S
23/59	HGG	2·0	>5	30 per cent in deposit; 10 per cent ~22·5S; 60 per cent ~11S
23/59	HGG	40	>5	Single peak in 12S

* This serum was from a rabbit which had proved immunologically responsive to HSA on an earlier occasion, and is not included in Table 1. The serum contained about 30 μg./ml. precipitating antibody.

ULTRACENTRIFUGAL BEHAVIOUR OF ANTIGEN–ANTIBODY COMPLEXES

Three sera containing only non-precipitating antibody and two containing precipitating antibody against HSA; three sera containing only non-precipitating antibody against BSA; and one serum containing non-precipitating antibody against HGG were examined. Each was mixed with a quantity of ^{131}I-labelled antigen much less than the antigen combining capacity together with ^{125}I-labelled marker proteins, and centrifuged through a sucrose density gradient as described in the section on Methods. The results are summarized in Table 2. In two of the antisera against HSA and one against BSA, the antigen–antibody complex sedimented as a homogeneous single peak, containing all the antigen, with sedimentation coefficient between 11 and 12 S. In the other antisera to HSA or BSA 25–95 per cent of the complexes sedimented to the bottom of the tube (S values 30 or greater), and the remainder occurred in peaks with approximate S values between 23 and 12·5. Typical sedimentation patterns are illustrated in Fig. 1. The possibility must be envisaged that antigen–antibody complexes dissociate during the course of sedimentation, and that the position of the antigen represents an equilibrium between sedimentation of a larger complex and its dissociation. However in view of the fact that a large excess of antibody was present, and that the smaller complexes did not sediment far enough to

become wholly separated from the γ-globulin, it is unlikely that significant dissociation occurred. This is to some extent confirmed by the symmetrical nature of the antigen distribution, and the fact that the whole of the antigen was present in a single peak.

It may be noted that in the sera 135/58 and 20/59, known to contain precipitating antibody, virtually all the antigen was found, as expected, in the deposit. The antiserum against HGG was tested at two concentrations of antigen. At 2 μg./ml. 30 per cent of the antigen was found in the deposit, while at 40 μg./ml. all the antigen appeared to sediment as a complex with sedimentation coefficient about 14 S.

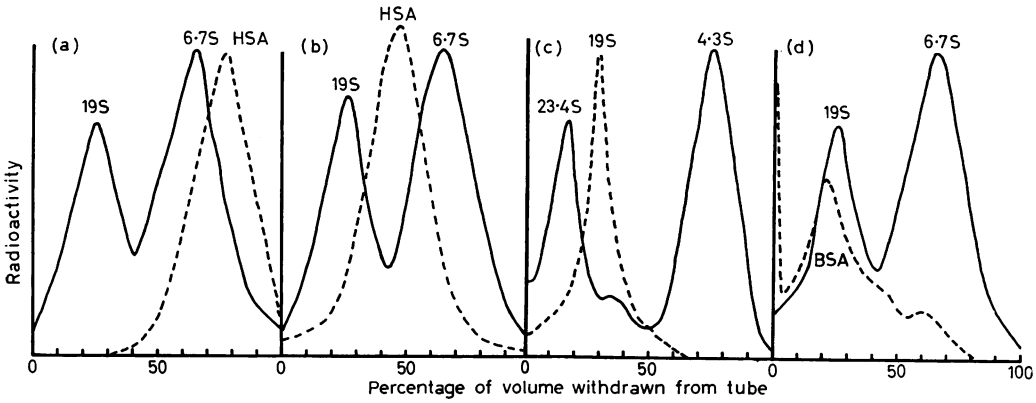

FIG. 1. Density gradient ultracentrifugation of antigen–antibody in antibody excess. (a) [^{125}I] human γ-macroglobulin, [^{125}I] HGG and [^{131}I] HSA in normal rabbit serum. (b) [^{125}I] human γ-macroglobulin, [^{125}I] HGG and [^{131}I] HSA in anti-HSA serum 17/59. (c) [^{131}I] haemocyanin (23·4S), [^{125}I] rabbit γ-macroglobulin and [^{131}I] BSA in normal rabbit serum. (d) [^{125}I] human γ-macroglobulin, [^{125}I] HGG and [^{131}I] BSA in anti-BSA serum 45/59. The density gradient consisted of 30 to 10 per cent sucrose in 0·1 M phosphate buffer, pH 7. Centrifugation was carried out for 15 hours at 25,000 rev./min. in the SW 40 rotor of a Spinco Model L Ultracentrifuge. Temperature reading about 10°.

The sedimentation coefficients listed in Table 2 are only approximate in most cases, but those given for the anti-HSA sera 131/58 and 17/59 are the means of three separate estimations, and that for the anti-BSA serum 100/58 the mean of two. The individual values all fell within 7 per cent of the respective means.

DIFFUSION COEFFICIENT AND MOLECULAR WEIGHT OF ANTIGEN–ANTIBODY COMPLEX

The results of the measurements of sedimentation coefficients of the various antigens in the presence of excess antibody indicated that, in some sera at least, very small complexes were formed. In order to calculate the molecular weight it was necessary to measure the diffusion coefficient also. Since to do this was somewhat time-consuming, a single serum, 17/59, was selected for study on the grounds that it had the highest antigen-combining power and consequently its antigen–antibody complexes might be expected to dissociate least during the process of measurement. Measurements of the diffusion coefficient of antigen were made as described under Methods at concentrations 3 and 9 μg./ml. serum. These gave identical results, indicating that excess antibody was present at both concentrations. Assuming that the diffusion coefficient of HSA at 20° is $6{\cdot}1 \times 10^{-7}$, the mean of four estimates of that of the complex was $3{\cdot}3 \times 10^{-7}$ (range $3{\cdot}02$–$3{\cdot}64 \times 10^{-7}$). The following calculations are due to Dr. P. A. Charlwood:

The molecular weight (M) is given by the Svedberg equation

$$M = \frac{RTS}{D(1-\bar{v}\rho)}$$

substituting $S_{20w} = 11{\cdot}65 \times 10^{-13}$, $D_{20w} = 3{\cdot}3 \times 10^{-7}$ and $\bar{v} = 0{\cdot}743$, and taking $R = 8{\cdot}314 \times 10^7$, $T = 293$ and $\rho = 0{\cdot}9982$ this gives a value $M = 330{,}000$ and a conventional frictional ratio $f/f_0 = 1{\cdot}40$.

These values may be compared with those which would be given by possible complexes of γ-globulin ($M = 150{,}000$, $\bar{v} = 0{\cdot}745$) and HSA ($M = 66{,}000$, $\bar{v} = 0{\cdot}736$) with molecular weights of the right order. Since antibody was in considerable excess, complexes containing more molecules of antigen than of antibody may be disregarded. The calculated values are given in Table 3, from which it will be seen that only those for a complex of one

TABLE 3

CALCULATIONS OF CONSTANTS FOR HYPOTHETICAL COMPLEXES OF ANTIBODY AND HSA

Complex	*M*	*v*	$S_{20w} \times 10^{13}$	$D_{20w} \times 10^7$	f/f_0
1 HSA+1 γ-globulin	216,000	0·742	11·65	5·02	1·07
1 HSA+2 γ-globulin	366,000	0·743	11·65	3·00	1·49
1 HSA+3 γ-globulin	516,000	0·744	11·65	2·14	1·86

molecule of HSA and two molecules of antibody agree with the experimental findings. The conclusion is therefore that with respect to antibody present in serum 17/59 the HSA contained two combining sites. This holds true irrespective of whether the antibody was bi- or uni-valent, since only one valency for antibody is involved.

REACTION OF ANTI-HSA SERA WITH THE HSA FRAGMENT

If the antisera against HSA combined with a limited number of combining sites on HSA, there was a reasonable likelihood that some sera would fail to react with a fragment representing only one-fifth of the molecule. This was tested in two ways, firstly by direct measurement of the combining power and secondly by observing the sedimentation behaviour of the fragment in the presence of the antisera. The combining capacities are set out in Table 4, which should be compared with Table 1 in which are recorded the combining capacities of the experimental sera with HSA. The four antisera against HSA, prepared by hyper-immunization of otherwise normal rabbits, all bound amounts of the fragment equal to one-fifth or more of the amounts of intact HSA which were precipitable by them. The two experimental sera (135/58 and 20/59) which contained traces of precipitating antibody also bound the fragment, though weakly in comparison with their capacity to bind HSA. So likewise did serum 40/59, which did not precipitate with HSA but formed relatively large complexes. By contrast the two sera (131/58 and 17/59) which formed very small complexes with HSA bound the fragment to a negligible extent, despite possessing considerable binding power for the whole molecule.

Experiments to measure the ultracentrifuge sedimentation characteristics of the fragment in the presence of excess antibody largely confirmed these results. The experiments were conducted in the same way as those described above, but the concentration of the

albumin fragment was 0·5 μg./ml. serum. In the presence of sera 131/58 or 17/59 the fragment behaved as in normal serum, giving a single peak with apparent sedimentation coefficient about 1S, whereas in the presence of serum 40/59 there were two peaks, 40 per cent of the fragment sedimenting with the 7S γ-globulin and the remainder having an

TABLE 4

BINDING OF HSA FRAGMENT BY VARIOUS ANTI-HSA SERA

Serum No.	*Precipitating antibody* (μg./ml.)	*Fragment-binding capacity* (μg./ml.) *in presence of antigen concn.:*				
		250 μg./ml.	100 μg./ml.	20 μg./ml.	5 μg./ml.	1 μg./ml.
Hyperimmune 1	13000	232	93	19	4·7	0·95
Hyperimmune 2	6100	126	85	18·5	4·7	0·95
Hyperimmune 3	9700	232	93	18·5	4·7	0·95
Hyperimmune 4	1000			15	4·6	0·95
35/58	0				0·1	0·03
131/58	0				0·1	0
135/58	30				0·9	0·15
17/59	0				0·15	0·06
19/59	0				0	0
20/59	72				0·9	0·2
38/59	0				0·17	0·07
40/59	0				1	0·4
42/59	0				0·15	0·1

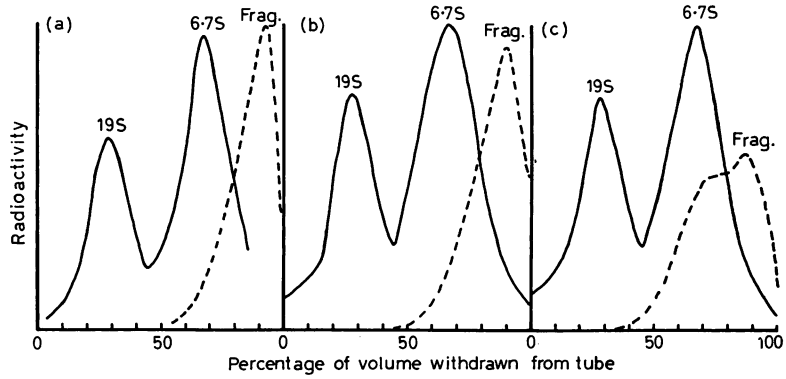

FIG. 2. Density gradient ultracentrifugation of HSA fragment. (a) [^{125}I] rabbit γ-macroglobulin, [^{125}I] monkey γ-globulin and [^{131}I] HSA fragment in normal rabbit serum. (b) The same mixture in anti-HSA serum 131/58. (c) The same mixture in anti-HSA serum 40/59. Conditions of centrifugation as in Fig. 1.

S_{20w} value about 1 (Fig. 2). In the presence of sera 135/58 and 20/59 most of the material had a sedimentation coefficient about 1S, but there was some distortion of the peaks in the direction of heavier components.

DISCUSSION

The findings presented above show that in respect of the three antigens HSA, HGG and BSA the immunological unresponsiveness which followed neonatal administration of antigen had only been very incompletely reversed at a time, many months later, when the capacity to make antibody had returned. This was evidenced in the first place by the fact

that the response to a course of antigen injections, which would have elicited a good response in normal rabbits, was very weak, and that in most animals such antibody as was produced would not precipitate with antigen. In other experiments, not recorded here, it was found that repetition of the administration of antigen (BSA in this instance) 1 month later to three rabbits caused no increase in nor altered the character of the antibody response.

In seeking an explanation for the failure of the antibodies to precipitate with their respective antigens it was found that the antisera formed relatively small complexes with the antigen, even in the presence of excess antibody. The failure to precipitate was therefore unlikely to be due primarily to formation of large but unusually hydrophilic complexes, but rather to restriction of the number of effective combining sites on the antigen molecules. In one of the seven non-precipitating antisera so examined the molecular weight of the complex was determined, and proved to be compatible with that to be expected for an antigen with only two combining sites. Since the complexes formed in two other antisera had similar sedimentation coefficients it is likely that in respect of these antisera also the antigen was at most bivalent. There was no evidence to indicate whether the two combining sites on the antigen were identical or different.

Study of the capacity of the anti-HSA sera to combine with a purified fragment of HSA, molecular weight 12,000, lends support to the idea that the number of sites with which the antibodies could combine was restricted. Thus comparison of the binding capacity of the sera for HSA at a concentration 10 μg./ml. (Table 1) and for the fragment at a concentration 5 μg./ml.—i.e. 2·5 times greater on a molar basis—(Table 4) shows that two sera which bound HSA quite strongly bound very little or none of the fragment. Three other such sera bound the fragment to an extent which, though definite, was markedly less than would have been expected from their combining power with HSA if they had behaved similarly to ordinary immune sera. Taken by itself this evidence only indicates that antibodies against the potentially antigenic sites on the fragment (which number at least two, and probably more since the fragment can precipitate with certain rabbit antisera), were represented poorly or not at all in the population of antibody molecules formed by the rabbits under investigation. It would clearly have been desirable to perform similar tests with several distinct purified fragments of each of the antigens if these had been available. However, taken with the other findings, the whole evidence is compatible with the hypothesis that rabbits which have been made immunologically unresponsive to an antigen recover the capacity to respond piecemeal, to one or two of the potentially antigenic sites at a time.

An alternative explanation for failure of the antibodies to precipitate with the antigens would be provided if the antibodies were univalent with respect to the antigens. Apart from the fact that all 7S rabbit antibodies examined from this point of view have proved to be bivalent, and that authentic instances of univalent rabbit antibodies have yet to be described, this hypothesis is unnecessary in view of the demonstrated deficiency of combining sites on the antigen in some of these systems. Furthermore the antibodies in some of the sera from rabbits which became responsive formed relatively large complexes, and in some the complexes were at least partly precipitable. This is much more likely to be due to the presence of a greater number of effective combining sites on the antigen in the latter sera, than to a difference from animal to animal in the valency of the antibodies.

Before any implications of these conclusions are discussed, a possible partial explanation of the findings must be considered. This is that the rabbits were undoubtedly all poor

responders, but in fact made antibodies against a variety of potentially antigenic sites on the antigens, and that the peculiar character of the antibody present in their sera was due to preferential combination of other antibodies with residual circulating antigen and selective removal of the complexes by the reticulo-endothelial system. In such consideration the timing of the antigen injections in relation to the onset and rate of immune elimination is important. ^{131}I-labelled soluble antigen was administered intravenously 1 week before a series of six injections of 2·5 mg. of alum-adsorbed antigen spaced over the next 2 weeks, and serum was taken 1 week after the last injection. It is impossible to state for how long the alum-adsorbed antigen remained in the circulation after each injection, but experience with particulate or denatured antigens in general would suggest a period of not more than an hour or so. The clearance of the labelled antigen was followed, however, throughout the whole period. From each of the rabbits which responded to the antigen, and whose sera were examined in these experiments, the labelled antigen had been eliminated between 12 and 17 days before the final serum samples were taken; furthermore there was no evident slowing of the rate of elimination consequent upon the injections of unlabelled alum-precipitated antigen, but rather the rate of elimination increased during the course of injections of these materials. This is not what would be expected if the alum-precipitated antigen had caused the removal of important quantities of precipitable antibody, and renders the explanation suggested above somewhat unlikely.

The very fact that immunological unresponsiveness is so specific—so that, for example, anti-BSA made in rabbits unresponsive to HSA does not cross-react with HSA, and *vice versa* (Humphrey, 1964)—implies that the unresponsiveness (just as the antibody response) must be determined separately by the various parts of the antigenic mosaic on a molecule rather than by the antigen as a whole. To this extent it is a reasonable hypothesis that, with the ending of unresponsiveness, the capacity to make antibody would return piecemeal in respect of different parts of the mosaic, and that the nature of the antibodies formed would vary from animal to animal according to the extent to which recovery had taken place. Furthermore it would follow that administration of more antigen might result in continued suppression of the response to some parts of the antigenic mosaic and stimulation of the response to others—a possibility discussed by Cinader and Dubiski (1963). The findings appear to favour such an hypothesis, and to provide a possible explanation of the 'partial tolerance' observed in respect of transplantation antigens (see, e.g., Hasek, Lengerova and Hraba, 1961).

Conclusions of this nature can be reached without making any detailed assumptions about the mechanisms by which or the sites at which immunological unresponsiveness is brought about in the first place, or how escape from it occurs. However, since antibodies against native proteins all combine with the intact undenatured molecules, they must be directed against antigenic configurations on the surface determined by the tertiary configuration of these molecules. This implies that the initiation of immune paralysis is an effect of whole molecules, or at least of fragments sufficiently large to maintain their tertiary structure intact and thus almost certainly to contain more than one of the potentially antigenic groupings. If both this and the general conclusions stated above are accepted, certain consequences may be deduced, differing according to the mechanism of paralysis which is considered. On the assumption that specifically paralysed cells persist in the body, and that they are temporarily blocked by the physical presence of antigen at key sites in the cell, then separate antigen molecules are required for each potential response which is blocked (and, should there be several such responses, the antigen molecules must persist

for varying lengths of time). If the block is due to secondary repressor mechanisms, then these must decay at varying rates. On the alternative assumption that paralysis involves either elimination or permanent blocking of the cells competent to respond to any part of the antigenic mosaic, it would follow that these cells must be replaced eventually by others which, in the early stages at least, have a restricted capacity to respond to different parts of the antigen.

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