THE EFFECT OF TOLERANCE ON THE SPECIFICITY OF THE ANTIBODY RESPONSE AND ON IMMUNOGENICITY

Antibody Response to Conformationally and Chemically Altered Antigens

By J. E. M. St. Rose,* Ph.D., and B. Cinader, D.Sc.

(From the Subdivision of Immunochemistry, Division of Biological Research, Ontario Cancer Institute, and the Departments of Medical Biophysics and Pathological Chemistry, University of Toronto, Toronto, Canada)

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Naturally acquired tolerance may be regarded as one of the mechanisms which regulate the antibody response. This regulation may be investigated by studying the response to antigens which have known homologies with autologous substances (1, 2), or by model experiments in which animals are rendered tolerant to a macromolecule and are then immunized with another but structurally related macromolecule. The question arises as to how the responsiveness of tolerant animals is related to the structural differences between tolerance-inducing and immunizing macromolecules and to what extent it is determined by inheritance.

Newborn animals injected with an antigen become immunologically unresponsive for a period which appears to vary with the amount of antigen given (3). During this period, an antibody response cannot be evoked by the tolerance-inducing antigen, but can be evoked by an antigen which is structurally related to it. This response of tolerant animals can be evoked by immunization with cross-reacting antigens, obtained by the coupling of synthetic determinants to the tolerance-inducing antigen (4–11). This finding has also been confirmed by experiments with homologous series of mammalian isofunctional proteins (12–15). In the latter studies, information on structural differences of the antigens is lacking so that an interpretation of experimental findings in terms of chemical differences could not be attempted. In the interpretation of the response of tolerant animals to chemically modified antigens, one can take into account that the difference between the tolerance-inducing and immunizing antigens may not be confined to the presence of the synthetic determinants, and that the introduction of synthetic determinants may affect the configuration of the protein. One might expect that the magnitude of this distortion would be related to the number of hapten groups per antigen molecule, as previously observed with oxazolonated compounds (16), and since this can be controlled, one should be able to investigate the effect on immunogenicity of conformationally changed determinants.

The response of tolerant animals is not entirely determined by the nature of the

* Present address: Defense Research Medical Laboratories, Downsview, Ontario, Canada.

cross-reacting antigen and some evidence for the involvement of hereditary factors will be presented.

In this paper we shall endeavor to explore the effect of tolerance on responsiveness to antigens which have been chemically modified to different extents and which have undergone consequent changes in molecular configuration. The specificity of the resulting antibody was examined by the method of hemagglutination-inhibition, using four antigens, HA, lightly and heavily diazotized HA, (HA-D_8, HA-D_{35}), and diazotized rabbit serum (RS-D). Erythrocytes sensitized with some of these antigens only react with a fraction of the total antibody. The properties of these antibody fractions can be examined by their reaction with different inhibitors which compete with the cell-attached antigen for antibody. The hemagglutination-inhibition technique permits us to detect antibody fractions which differ in specificity and also to detect different classes of antigenic determinants.

Materials and Methods

Reagents

Reagents used in the determination of nitrogen by Micro-Kjeldahl were M.A.R. grade. All reagents were made up in glass-distilled water.

Human Albumin (HA).—Human albumin, Behringwerke "reinst" (Behringewerke, Marburg-Lahn, West Germany) was used throughout.

2-Mercaptoethanol was obtained from Eastman Organic Chemicals, Rochester, N. Y.

Radioactive Isotopes.—Iodine was obtained as cysteine-free sodium radioiodide Na ^{131}I from Charles E. Frosst, Montreal, Quebec, Canada. ^{35}S-labeled sulfanilic acid was obtained from the Radiochemical Centre, Amersham, England.

Tannic acid was obtained from British Drug Houses, Toronto, Canada (batch 16465).

Agarose was obtained from Industries Biologique, Gennevilliers, France.

Glycine Buffer (pH 8.4).—The buffer consisted of 14.260 g glycine, 11.115 g NaCl, and 100 ml 0.1 M NaOH, made up to 2 liters with glass-distilled water.

Buffered Agarose Gel (1.1% w/v agarose). The gel was prepared by adding to 1 liter of glass-distilled water, 2 g sodium azide, and 24 g agarose. This mixture was kept at 100°C for 45 min, and was mixed at 56°C with 1 liter of filtered glycine buffer.

Analytical and Immunological Methods

Immunoelectrophoretic Analysis (*I.E.A.*).—I.E.A. was carried out in an LKB-Produkter apparatus (6800A). Antisera were allowed to migrate for 35 min in buffered agarose gel. The potential difference was 250 v and the current was 16 ma.

Optical Rotatory Dispersion (*ORD*).—ORD measurements were carried out in a Jasco ORD/UV-5 spectropolarimeter.

Determination of Radioactive Counts.—^{35}S analyses were carried out in a gas-flow window counter, Nuclear-Chicago model 181A, Nuclear-Chicago Corp., Des Plaines, Ill. ^{131}I counts were obtained using an automatic scintillation well-counter, Nuclear-Chicago model C120.

Preparation of Modified Proteins, Azo Human Albumin (*HA-D*), *and Azo Rabbit Serum* (*RS-D*).—Diazotized sulphanilic acid coupled to human serum albumin was prepared as previously described (4, 5). Three products were prepared which differed in the degree of coupling (Table I). The quantity of *p*-azosulfonic acid per unit quantity of protein was calculated from

the ratio between the radioactivity and weight of the *p*-azosulfonic acid and the corresponding ratio of the conjugated protein. The molar ratio was then determined on the basis of known molecular weights of *p*-azosulfonic acid and of human albumin.

To prepare RS-D, diazonium salt was added in the proportion of 0.05 mg sulfanilic acid per 1 mg of rabbit serum protein.

Iodinated Human Albumin (*HA*.^{131}*I*).—Iodination was carried out by a modification (17) of the method of Berson, Yalow, Schrieber, and Post (18).

Induction of Tolerance in Newborn Rabbits.—Newborn rabbits were injected intraperitoneally with HA dissolved in 0.15 M NaCl: the first injection was given within 12 hr of birth. A total of 4 × 5 mg of HA was administered within 84 hr after birth.

Injection of Adult Rabbits.—Injections of protein dissolved in 0.15 M NaCl were made into the marginal vein of the ear. A course of injections consisted of three injections administered at intervals of 48 hr. The rabbits were injected intravenously with HA-D on days 44, 46, 48, 69, 71, and 73; animals were bled on days 54, 59, 78, and 84. Rabbits were finally injected with HA.^{131}I (5.24 mg/kg body weight) on day 85 and were thereafter bled daily.

TABLE I

Properties of Native and Conjugated Human Albumin

Designation of protein and derivatives	No. of *p* azosulfonic acid groups per molecule of protein	Amount of sulfanilic acid added per mg of protein	Mean residue rotation (degrees cm per decimole) at 234 mμ	Electrophoretic migration in cm*	Absorbance at λ = 342 mμ‡
		mg			
HA	0	0	−10,300	1.87	0.008
HA-D_8	8	0.051	−9,700	2.23	0.395
HA-D_{16}	16	0.102	n.d.	n.d.	n.d.
HA-D_{35}	35	0.206	−9,000	2.99	1.155

n.d., not done.

* Distance from starting position towards anode.

‡ Protein (50 μg N/ml) was dissolved in phosphate buffer pH 8.5.

Agglutination (*4, 19, 20*).—Sheep erythrocytes were washed three times with 0.15 M NaCl, suspended in phosphate-saline buffer (pH 7.2) to give a 2.5% suspension, and were then mixed with an equal volume of tannic acid (1/30,000) in 0.15 M NaCl. After 10 min the tanned erythrocytes were centrifuged, washed with phosphate buffer at pH 7.2, and suspended in 0.15 M NaCl. The concentration of this suspension of tanned cells was so adjusted that when 0.5 ml was added to 7.5 ml of distilled water and treated with a mixture of 5% carbon monoxide and 95% nitrogen for 2 min the optical density of the resulting solution was 0.3 (Coleman Junior Spectrophotometer at λ = 540) in a tube 1.0 cm in diameter. To 10 ml of this suspension were added 40 ml phosphate buffer pH 6.4 containing 2.5 mg of antigen. After 15 min the tanned antigen-coated cells were centrifuged and washed three times with a 1.0% (v/v) solution of normal rabbit serum in 0.15 M NaCl. The concentration of these tanned coated cells was adjusted as before. Antisera for hemagglutination tests were heated in stoppered tubes at 56°C for 60 min. Natural sheep antibodies were absorbed for 1 hr with 0.1 ml of washed packed sheep erythrocytes per 1.0 ml of serum, and the erythrocytes were then removed by centrifuging. Sera were diluted in 1% normal rabbit serum in 0.15 M NaCl, and to each of the serum dilutions was added one drop of the suspension of tanned coated sheep erythrocytes

from a pipette calibrated to deliver 0.05 ml per drop. A low titer, a high titer, and a normal serum were included in all tests; tests were rejected if the titer of these standard sera deviated from a previously established norm.

Examination of the Specificity of Antibodies by Agglutination-Inhibition.—This method has been applied in earlier studies of acquired immunological tolerance (4, 5). Tanned sheep erythrocytes were coated with azoproteins and were agglutinated with antibody formed in response to these azoproteins. The agglutination titer was reduced if soluble antigens were allowed to react with the immune serum before tanned coated cells were added. Different quantities of antigen dissolved in 0.15 M NaCl, 0.15 ml of immune serum, and a quantity of 1% normal rabbit serum in 0.15 M NaCl were made up to give a final volume of 1.5 ml. The mixtures were incubated for 1 hr at 37°C, then kept at 2° ± 1°C overnight. The agglutination titers of the immune sera were subsequently examined and compared with those of sera to which antigen had not been added.

Determination of the Agglutinating Capacity of Antisera after Preincubation with 2-Mercaptoethanol.—The procedure was based on that adopted by Uhr and Finkelstein (21). Serum was diluted 1:10 with 0.15 M NaCl and incubated in a final concentration of 0.1 M 2-mercaptoethanol at 37°C for 30 min. The agglutination titer was then determined in the presence of mercaptoethanol using tanned erythrocytes coated with HA-D. As a control, the titer of untreated sera was measured in the same experiments.

Elimination Tests.—Animals to be injected with iodinated albumin were given KI in their drinking water during the week preceding the injection. Iodinated albumin was injected into the marginal vein of one ear and blood was taken from the opposite ear at intervals of 24 hr. The radioactivity of the sera was determined before and after treatment with trichloroacetic acid. This treatment consisted of adding an equal volume of 20% trichloroacetic acid to the serum, centrifuging at 2500 rpm for 30 min, and then determining the activity of the precipitate.

RESULTS

Animals tolerant to human albumin and normal animals were injected with derivatives of human albumin (HA) which had been coupled with varying numbers of diazonium groups. Such protein derivatives thus resemble one another in having the same hapten but they differ markedly from one another in the extent to which the protein structure has been distorted. The period during which the animals were injected with the modified proteins was restricted to the period during which tolerance is maintained.

The proportion of tolerant animals which responded to injections of various azo derivatives of HA was determined. The specificity of the antibodies thus induced was examined by agglutination-inhibition tests. For this purpose we employed tanned cells sensitized with antigens that differed in the extent of conformational alteration and therefore reacted with different fractions of the total antibody. These fractions were characterized by the inhibitory capacity of the same series of reagents. The effect of injection of chemical derivatives on the tolerance to HA was also evaluated in terms of the elimination of lightly iodinated human albumin (HA.^{131}I) from the rabbits' blood stream.

The Duration of the Tolerant State.—Newborn rabbits were given, during the first 84 hrs after birth, four injections of HA, each of 0.7 mg N. Different groups of these animals were thereafter given a single injection of HA.^{131}I. Animals

which eliminated the protein in two phases were considered tolerant, and animals which eliminated the protein in three phases were considered to have lost tolerance. The percentage of tolerant animals was plotted as a function of the age at which they had been injected with HA.^{131}I (Fig. 1). A considerable heterogeneity was observed in the duration of tolerance. Most animals however, maintained tolerance for 80 days.

We next established that antibody could not be elicited in animals injected with HA at birth if they were given HA up to the 73rd day of life. Rabbits injected with HA at birth were given two courses of three injections of HA between the 44th and 73rd day of life. None of these animals made any antibody

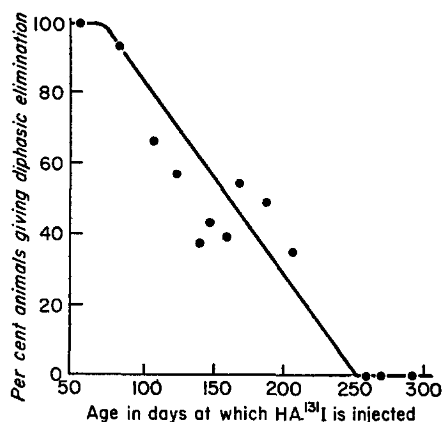

Fig. 1. The duration of acquired immunological tolerance to HA in the rabbit. Animals were injected at birth with HA (2.8 mg N) and were left untreated thereafter, except for one injection with 7.0 μg N per kg body weight of lightly iodinated human albumin (HA.^{131}I). The per cent of animals showing diphasic elimination was plotted as a function of the age at which HA.^{131}I was injected.

detectable by agglutination of tanned and sensitized erythrocytes (top line Table II).

Incidence of Antibody Response in Tolerant Animals.—On the basis of the above experiments, all immunizations with HA-D were terminated within 73 days. We immunized normal controls and tolerant animals with different *p*-azosulfonic acid derivatives of human albumin. The sera were examined for their capacity to agglutinate tanned erythrocytes sensitized with the azo derivative with which the animal had been injected, and also with tanned erythrocytes sensitized with HA. This test showed that 92% (11/12) of the control animals immunized with HA-D_8, and 100% (12/12) of the animals immunized with HA-D_{35} made antibody which agglutinated tanned red cells sensitized with HA as well as tanned red cells sensitized with the immunizing antigen.

Of the tolerant animals immunized with azo derivatives of HA, 24% (35/143) made antibody detectable with tanned erythrocytes sensitized with HA-D. The incidence of antibody producers was higher in the groups of rabbits immunized with HA-D_{35} than in those immunized with HA-D_8. Antibody which agglutinated tanned erythrocytes sensitized with HA was found in the sera of 15% (21/143) of tolerant animals immunized with azo derivatives.

The sera from the different groups of animals were titrated for their content of antibody capable of agglutinating erythrocytes coated with unmodified HA.

TABLE II

Response of Rabbits, Tolerant to HA, Which Have Been Given Injections of HA-D During Adult Life

Animals were injected with 20 mg human albumin (HA) within the first 84 hr of birth, and were given two courses consisting of six injections of *p*-azosulfonic acid human albumin (HA-D). Animals were injected with lightly iodinated human albumin (HA.^{131}I) 12 days after the last injection.

No. of *p*-azosulfonic acid groups (D) per molecule of albumin	Amount of azosulfonic acid in each injection	Amount of HA-D in each injection	Fraction of animals making antibody which caused agglutination of tanned cells sensitized with		Fraction of animals showing immune elimination of HA.^{131}I
			HA-D	HA	
	mg	*mg*			
0	—	5.0	0/20 (0%)*	0/20 (0%)	0/3
8	0.10	5.0	10/58 (17%)‡	8/58 (14%)	0/11
16	0.20	5.0	2/12 (17%)§	0/12 (0%)	—
35	0.10	1.1	9/36 (25%)‖	7/36 (19%)	—
35	0.48	5.0	14/37 (38%)¶	6/37 (12%)	0/11

* compared with ‡ $0.025 < P < 0.05$; with § $0.05 < P < 0.10$; with ‖ $0.01 < P < 0.025$; with ¶ $P < 0.005$.

‡ compared with § $P > 0.90$; with ‖ $0.25 < P < 0.50$; with ¶ $0.01 < P < 0.025$.

§ compared with ‖ $0.25 < P < 0.50$; with ¶ $0.10 < P < 0.25$.

‖ compared with ¶ $0.75 < P < 0.90$.

Reciprocal agglutination titers were lower in the sera of tolerant animals than in the sera of corresponding controls (for animals treated with HA-D_8, $0.05 < P < 0.10$, and for animals treated with HA-D_{35}, $P < 0.001$). Sera from control animals immunized with HA-D_8 gave lower titers than sera of control animals immunized with HA-D_{35} ($P < 0.001$) (Table III). However, in the tolerant group, titers were higher in HA-D_8 sera than in HA-D_{35} sera ($0.025 < P < 0.05$).

Specificity of Antibody to HA-D_8 and HA-D_{35}.—We shall consider first the response of control animals which had *not* been injected with HA at birth and which were given six injections with HA-D_8 and HA-D_{35}. Agglutination-inhibition tests were carried out with tanned sheep erythrocytes coated with the

immunizing antigen. Antibody produced in response to HA-D_8 could be completely inhibited by HA-D_8, and could be partially inhibited by HA (Fig. 2). The agglutinating capacity of most sera was reduced substantially with 1–10 μg N HA. With six of the nine sera the agglutinating capacity could be reduced

TABLE III

Antibody Response to Immunization with HA-D of Normal Animals and of Animals Injected with HA at Birth

Antibody measured by agglutination of tanned red cells sensitized with HA. All animals were given six injections (0.7 mg N each) of HA-D.

Immunizing antigen	Control animals (not injected at birth)		Tolerant animals (injected at birth)		*P*
	Reciprocal agglutination titers	m* ± SD	Reciprocal agglutination titers	m* ± SD	
HA-D_8	20,000	8050 ± 8900	1050	1050 ± 950	$0.05 < P < 0.1$
	2,000		100		
	9,000		500		
	25,000		2750		
	200		650		
	1,500		700		
	6,150		100		
	450		2500		
HA-D_{35}	26,250	32,600 ± 12,000	50	150 ± 80	$P < 0.001$
	20,000		250		
	53,350		200		
	16,000		50		
	27,500		200		
	21,000		50		
	36,000				
	40,000				
	35,000				
	50,000				
	$P < 0.001$		$0.025 < P < 0.05$		

* m, arithmetic mean.

further by considerable antigen excess and with one serum, it could be abolished completely.

Antibody produced in response to HA-D_{35} could be completely inhibited by HA-D_{35}. In all cases but one, excess of HA also inhibited completely the capacity to agglutinate (Fig. 3).

It thus appears that at least a proportion of the antibody elicited by HA-D_8

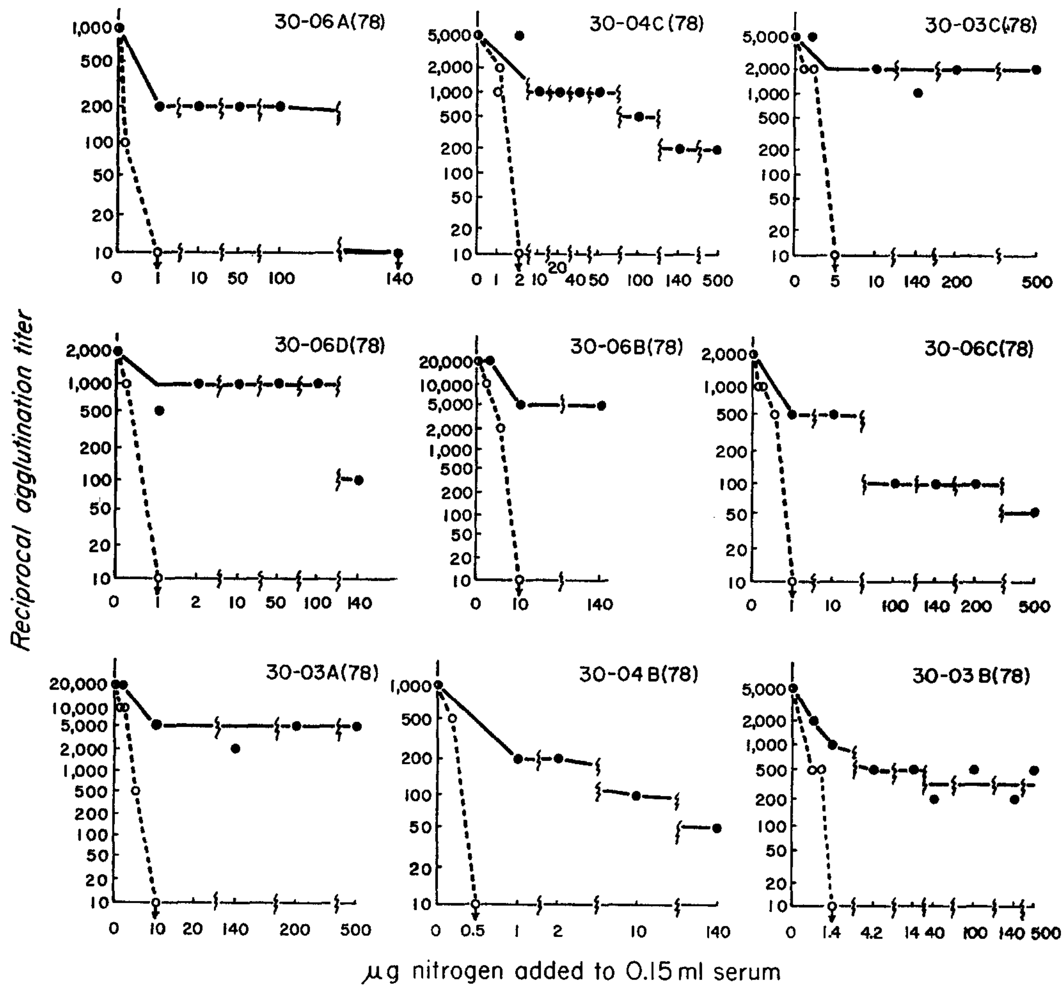

FIG. 2. Specificity of antibody to HA-D_8 from normal rabbits *not injected* at birth with HA but given six injections of HA-D_8 in later life. Agglutination of tanned cells sensitized with HA-D_8. Agglutination-inhibition carried out with HA or HA-D_8. Number above each graph indicates designation of rabbits, number in brackets gives the age, in days, of the animal from which the serum was obtained. - - - -O- - - -, reciprocal titer after inhibition with HA-D_8; and ——●——, reciprocal titer after inhibition with HA. Arrows indicate reciprocal titer less than 10.

was directed predominantly to the azo determinants. On the other hand, the antibody produced in animals immunized with HA-D_{35} could be completely, though inefficiently inhibited with HA and seemed therefore, to be directed to a considerable extent to configurationally altered protein determinants of

HA-D_{35} or to determinants which included the hapten as well as the surrounding amino acids.

The same type of analysis was applied to the sera from tolerant animals. The agglutinating capacity of the antibody elicited by HA-D_8 could be completely inhibited by HA-D_8, and could be partially inhibited by HA (Fig. 4).

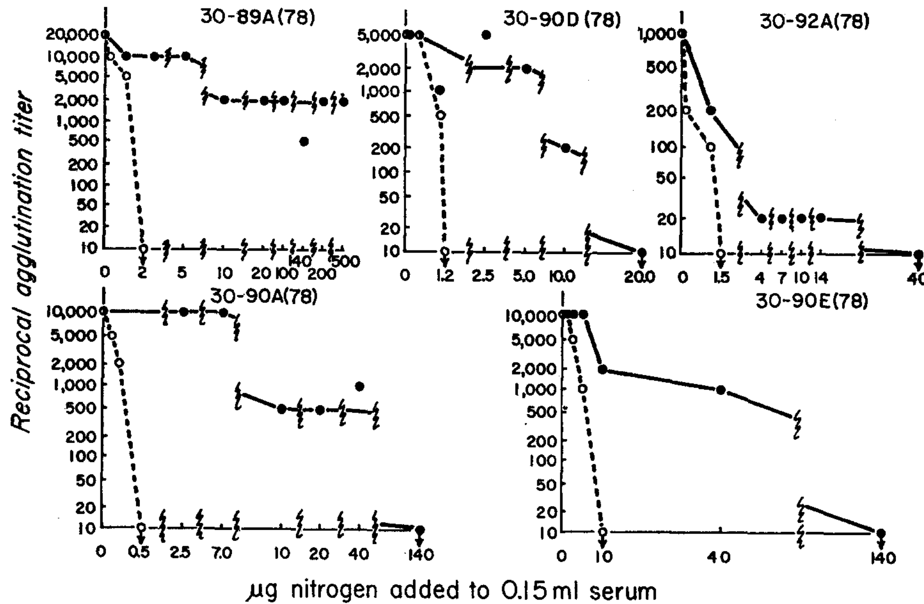

FIG. 3. Specificity of antibody to HA-D_{35} from normal rabbits *not injected* at birth with HA but given six injections of HA-D_{35} in later life. Agglutination of tanned cells sensitized with HA-D_{35}. Agglutination-inhibition carried out with HA or HA-D_{35}. Number above each graph indicates designation of rabbits, number in brackets gives the age, in days, of the animal from which the serum was obtained. ---O---, reciprocal titer after inhibition with HA-D_{35}; and ——●——, reciprocal titer after inhibition with HA. Arrows indicate reciprocal titer less than 10.

The reduction in agglutinating capacity brought about by corresponding quantities of HA (adjusted to the reciprocal agglutination titer of the antisera) was approximately half of that observed in controls.

The specificity of antibody obtained from tolerant animals that had been immunized with HA-D_{35} differed markedly from that of sera from corresponding control animals as well as from that of tolerant animals immunized with HA-D_8. The agglutinating capacity of HA-D_{35} antisera from tolerant animals could be readily abolished by preincubation of the antisera with HA-D_{35}, but was not reduced by quantities of HA which substantially reduced the agglutination titer of antisera from the corresponding controls. The agglutination titer of two

of the antisera from tolerant animals, though unaffected by as much as 50 μg N HA/0.15 ml could be somewhat reduced by excess of HA (Fig. 5).

Thus the response of tolerant animals immunized with HA-D_8 and with

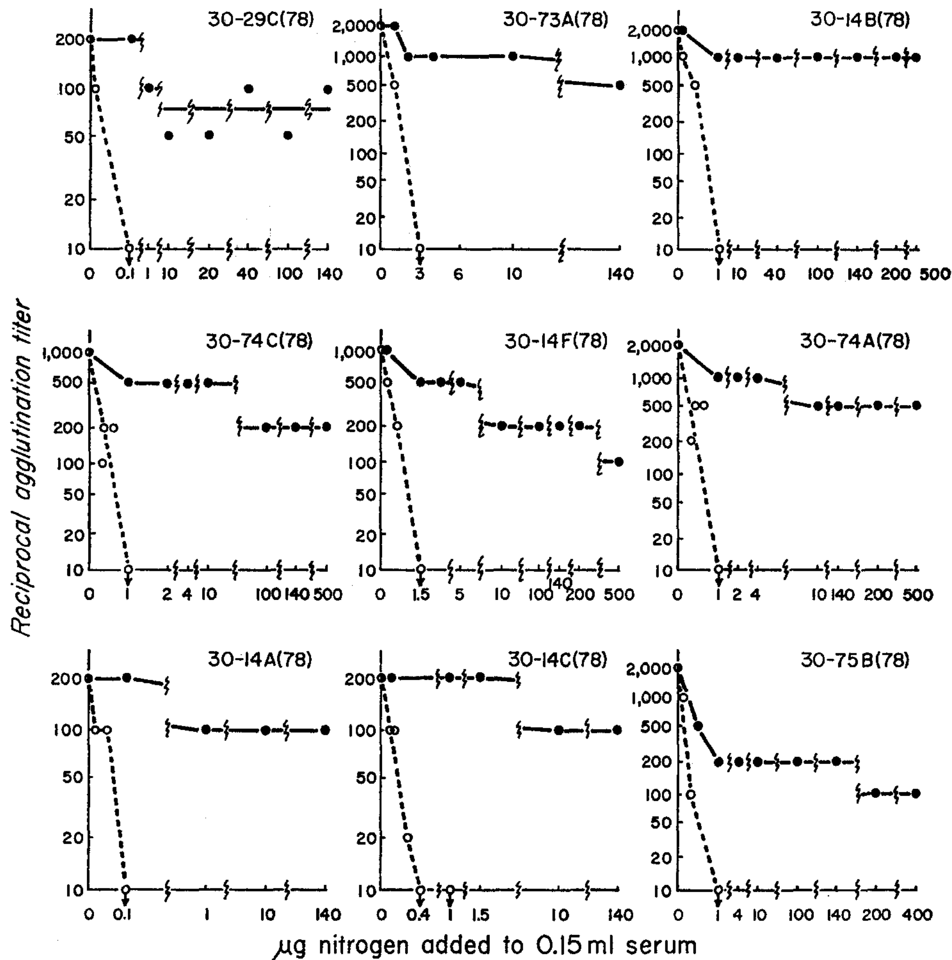

FIG. 4. Specificity of antibody to HA-D_8 from rabbits *injected* with HA at birth and subsequently given six injections of HA-D_8. Agglutination of tanned cells sensitized with HA-D_8. Agglutination-inhibition carried out with HA or HA-D_8. Number above each graph indicates designation of rabbits, number in brackets gives the age, in days, of the animal from which the serum was obtained. - - -○- - -, reciprocal titer after inhibition with HA-D_8; and ——●——, reciprocal titer after inhibition with HA. Arrows indicate reciprocal titer less than 10.

HA-D_{35} differed from the response of normal animals in the incidence of antibody formers, in the titer of the sera of these animals, and in the specificity of the antibody.

The remarkable differences between the response of tolerant animals to HA-D_8 and HA-D_{35} would be difficult to understand if one regarded the two azo compounds as consisting of the same determinants and differing only in the number of determinants. However these differences become understandable if one assumes that there is in addition to chemical modification, a change in the folding of the peptide chains. In fact, there is a change in the optical rotatory

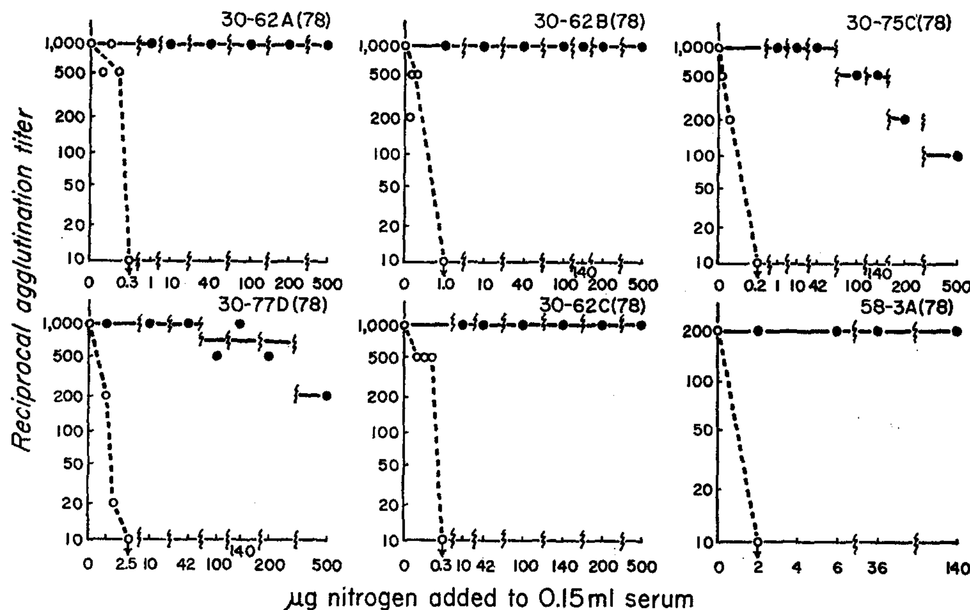

FIG. 5. Specificity of antibody to HA-D_{35} from rabbits *injected* with HA at birth and subsequently given six injections of HA-D_{35}. Agglutination of tanned cells sensitized with HA-D_{35}. Agglutination-inhibition carried out with HA or HA-D_{35}. Number above each graph indicates designation of rabbits, number in brackets gives the age, in days, of the animal from which the serum was obtained. - - -O- - -, reciprocal titer after inhibition with HA-D_{35}; and ——●——, reciprocal titer after inhibition with HA. Arrows indicate reciprocal titer less than 10.

dispersion (O.R.D.) following the introduction of 8 azo groups per molecule of HA, and a still greater change on the introduction of 35 azo groups (Table I). In interpreting our findings we must, therefore, take into account that conformational change may have produced new protein determinants in HA-D to which animals injected at birth with native HA may not be tolerant and that these determinants will be more abundant or more prominent in the conformationally more profoundly altered HA-D_{35}. We may thus expect that antibodies may be elicited by conformationally altered determinants and that the specificity and abundance of these antibodies would not be identical in animals immunized with HA-D_8 and HA-D_{35}. Further insight into this may be obtained

from agglutination-inhibition tests in which both types of azo compounds are employed to coat cells and to inhibit the agglutination reaction.

We shall first examine antisera from animals which were immunized with HA-D_8, employing tanned cells coated with HA-D_8, and shall begin with an analysis of antibody from animals not rendered tolerant (Fig. 6, upper left).

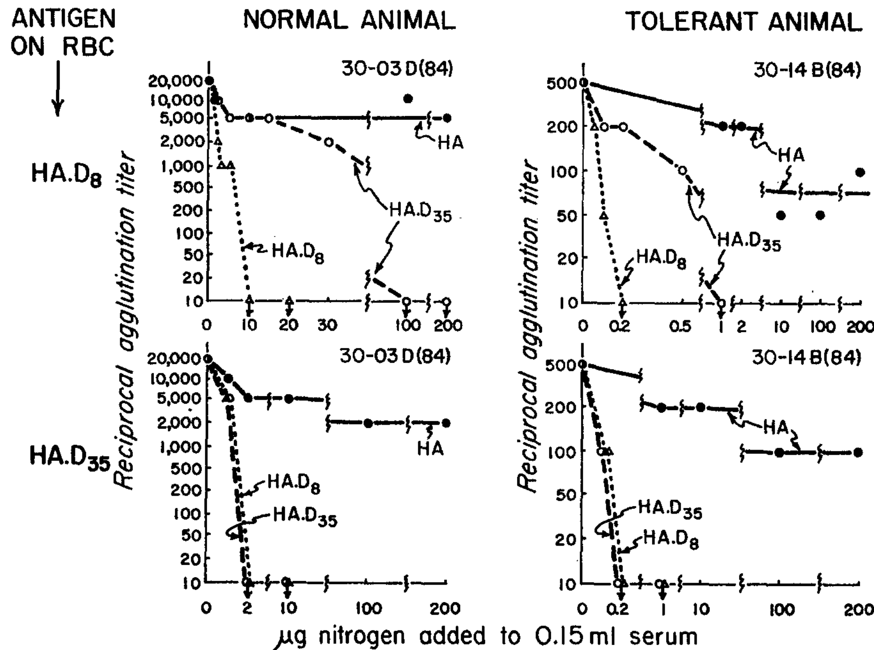

FIG. 6. The relative inhibitory capacity of HA, HA-D_8 and HA-D_{35}; the specificity of HA-D_8 antibody. All sera were obtained by immunization with HA-D_8. Agglutination-inhibition experiments were carried out with tanned erythrocytes sensitized with HA-D_8 (curves in upper part of graph) and tanned erythrocytes sensitized with HA-D_{35} (curves in lower part of graph). The inhibitory capacity of HA, HA-D_8, and HA-D_{35} were compared. Curves on the left hand side show inhibition of serum from a normal rabbit, and curves on the right hand side show inhibition of serum from a tolerant rabbit. Number above each graph shows the number of the animal and, in brackets the age, in days, of the animal from which the serum was obtained. - - -○- - -, reciprocal titer after inhibition with HA-D_{35}; - - -△- - -, reciprocal titer after inhibition with HA-D_8; and ——●——, reciprocal titer after inhibition with HA. Arrows indicate reciprocal titer less than 10.

Agglutination by these antibodies could be inhibited completely with HA-D_8 and HA-D_{35}, but more of the latter compound than of HA-D_8 was required for a given decrease in agglutinating capacity. It follows that both antigens contain determinants which can combine with all the antibody molecules, but that there are structural differences between the determinants such that HA-D_{35} combines

less firmly than HA-D_8 with all or some of the antibody molecules. To test the validity of this conclusion, agglutination-inhibition tests were next carried out with tanned red cells sensitized with HA-D_{35} (Fig. 6, lower left). The inhibitory efficiency of HA-D_8 and HA-D_{35} were now identical. Presumably the fraction

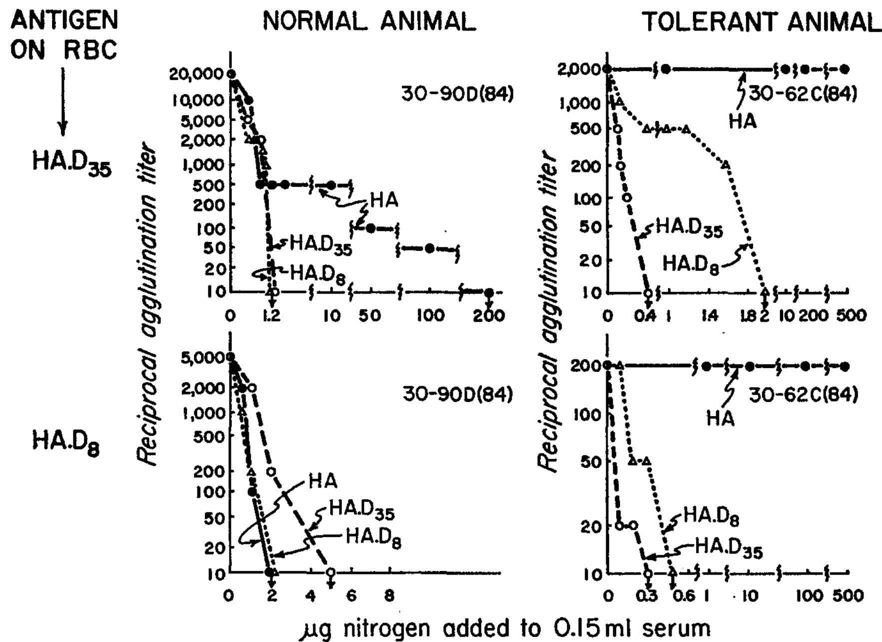

FIG. 7. The relative inhibitory capacity of HA, HA-D_8, and HA-D_{35}; the specificity of HA-D_{35} antibody. All sera were obtained by immunization with HA-D_{35}. Agglutination-inhibition experiments were carried out with tanned erythrocytes sensitized with HA-D_{35} (curves in the upper part of graph) and with tanned erythrocytes sensitized with HA-D_8 (curves in lower part of graph). The inhibitory capacity of HA, HA-D_8, and HA-D_{35} were compared. Curves on the left hand side show inhibition of serum from a normal rabbit, and curves on the right hand side show inhibition of serum from a tolerant rabbit. Number above each graph shows the number of the animal and, in brackets the age, in days, of the animal from which the serum was obtained. - - -O- - -, reciprocal titer after inhibition with HA-D_{35}; - - -△- - -, reciprocal titer after inhibition with HA-D_8; and ——●——, reciprocal titer after inhibition with HA. Arrows indicate reciprocal titer less than 10.

of the antibody which combines with HA-D_{35}-coated red cells reacts equally efficiently with HA-D_8 and HA-D_{35}. It seems reasonable to conclude that structural differences in the determinants of HA-D_8 and HA-D_{35} render the determinants of HA-D_{35} relatively inefficient in competition for antibody to HA-D_8. The specificity analysis of antibody from tolerant animals immunized with HA-D_8 gave essentially the same experimental results as that of the antibody

from control animals (Fig. 6, right). The results of this specificity analysis does not allow us to decide whether the antibody to HA-D_8 is directed to conformationally altered protein determinants, to the azo groups, or to a determinant consisting of azo groups and of surrounding amino acids (Fig. 6). We shall return to this point later.

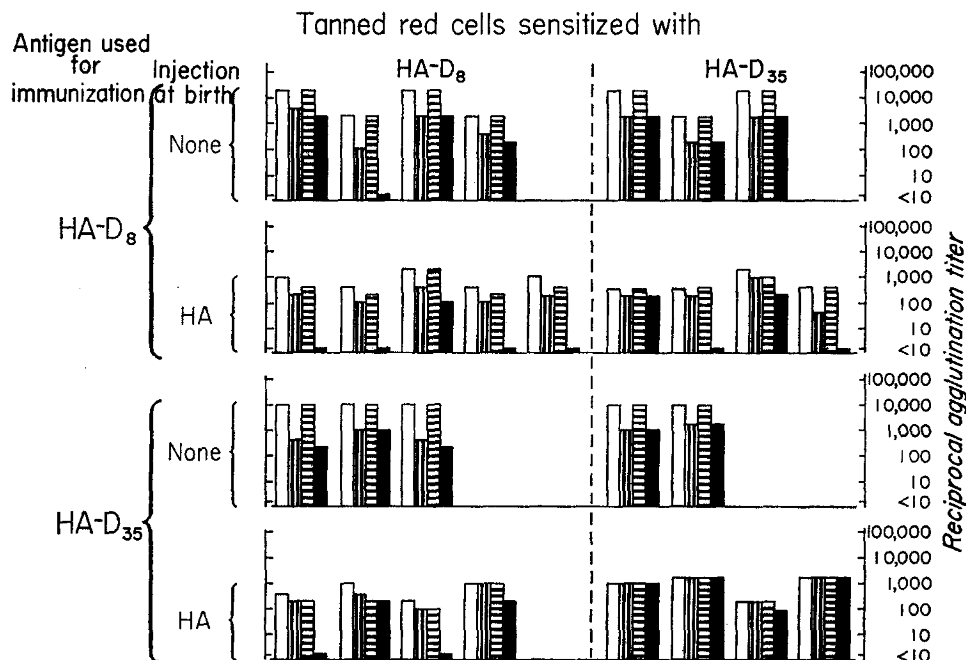

FIG. 8. Inhibitory capacity of azo rabbit serum (RS-D). Agglutinations were carried out with tanned erythrocytes sensitized with HA-D_8 and HA-D_{35}. The reciprocal titer of sera from normal and from tolerant animals was determined before and after incubation with HA, RS-D, and with a mixture of HA and RS-D. □, reciprocal titer in the absence of inhibitor; ▥, reciprocal titer after inhibition with 200 μg N HA/0.15 ml of serum; ⊟, reciprocal titer after inhibition with 200 μg N RS-D/0.15 ml of serum; and ■, reciprocal titer after inhibition with a mixture of 200 μg N HA and 200 μg N RS-D/0.15 ml of serum.

Agglutination-inhibition tests were next applied to antibody obtained from animals immunized with HA-D_{35}. We shall again give first attention to the antibody from control animals. When cells coated with HA-D_{35} were employed, HA-D_8 and HA-D_{35} were equally effective inhibitors in agglutination-inhibition tests; HA was a relatively inefficient inhibitor since nearly 200 times more HA than HA-D was needed to prevent agglutination completely (Fig. 7). If the agglutination-inhibition was examined with HA-D_8-coated cells, a different relationship between the inhibitory capacity of the three antigens was found.

In this case HA-D_8 and HA were equally effective inhibitors whereas HA-D_{35} was less effective (2½ times).

It would seem that agglutination-inhibition tests with cells sensitized with HA-D_8 reveal the properties of a subfraction of the total antibody, which is

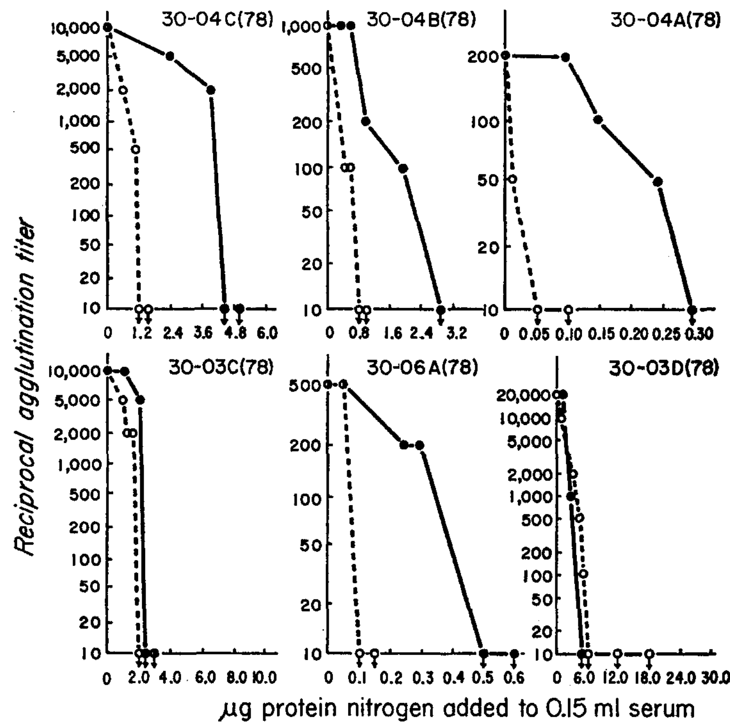

FIG. 9. The specificity of antibody to protein determinants formed by animals *not injected* at birth in response to immunization with HA-D_8. Sera were obtained from animals not injected at birth and given six injections of HA-D_8 (0.7 mg N each) between day 44 and day 73 after birth. Tanned sheep erythrocytes sensitized with HA, were agglutinated by 11/12 sera. The agglutinating capacity of these antisera was inhibited by prior addition of HA-D_8 and HA. Number above each graph indicates designation of rabbits, number in brackets gives the age, in days, of the animal from which the serum was obtained. ----O----, inhibition by HA-D_8; and ——●——, inhibition by HA.

directed against protein determinants present on HA and on HA-D_8 but are conformationally so altered on HA-D_{35} as to make this antigen an inefficient inhibitor. Thus we may conclude that several antibody fractions of different specificity can be discerned and that some are directed to determinants which are shared between HA-D_8 and HA-D_{35}, and others to determinants which are shared between HA-D_8 and HA.

With this background information we can now employ agglutination-inhibition tests to examine sera from tolerant animals immunized with HA-D_{35}. In this case no inhibition could be observed with HA whether tanned cells sensitized with HA-D_8 or HA-D_{35} were employed (Fig. 7). The degree of inhibition by HA-D_8 and by HA-D_{35} was approximately the same with both types of sensitized cells, and HA-D_{35} was always the more efficient inhibitor.

Thus the fraction of antibody was absent, which in the sera of controls reacted with determinants shared by HA and HA-D_8, and the results were compatible with the view that the largest part of the antibody was directed against determinants which consisted of the azo group and the conformationally altered surrounding area of the protein.

Reaction with Rabbit Serum, Conjugated with P-Azosulfonic acid (RS-D).—We have thus shown that at least two types of specificity may be distinguished, in the antibody made by animals tolerant to HA following immunization with HA-D; one type of antibody which was directed against azo determinants and surrounding moieties of the protein molecule, and another type which was directed against conformationally altered protein determinants. In an attempt to gain further insight into the specificity of these antibodies, the following experiment was undertaken. Agglutination-inhibition tests were carried out with tanned red cells sensitized with HA-D_8 or HA-D_{35} and the inhibitory effect of RS-D was examined. Antibody formed by tolerant animals in response to HA-D_8 and HA-D_{35} was only slightly or not at all inhibited by as much as 200 μg N RS-D/0.15 ml of serum whether HA-D_8 or HA-D_{35} was on the tanned red cell (Fig. 8). However, with cells sensitized with HA-D_8 a marked reduction in titer could be seen in 8/9 animals when the same quantity of RS-D was added to sera in the presence of HA (200 μg N) (Fig. 8). With the serum obtained from tolerant animals immunized with HA-D_8 this reduction in titer was found whether tanned cells sensitized with HA-D_8 or HA-D_{35} were employed. On the other hand, with serum from tolerant animals immunized with HA-D_{35} the results differed markedly if tanned cells sensitized with HA-D_{35} rather than with HA-D_8 were employed. With the former reagent, the serum from tolerant animals immunized with HA-D_{35} was not inhibited by a mixture of RS-D and HA. It appeared therefore that a fraction of antibody was formed by tolerant animals in response to HA-D_{35} which was not formed by tolerant animals in response to HA-D_8. O.R.D. measurements have indicated that the degree of conformational change was larger in HA-D_{35} than in HA-D_8 and it seemed reasonable that the fraction of antibody, which was absent in antisera to HA-D_8 and present in antisera to HA-D_{35}, was directed against conformationally altered protein determinants.

Reaction with the Tolerance-Inducing Molecule (HA).—Having so far examined the specificity of antibodies with cells sensitized with various azo compounds, we turned next to an analysis of the specificity of that fraction of the

antibody which could agglutinate tanned cells coated with HA. The quantities of this antibody formed upon immunization of tolerant animals with HA-D_{35} were so small that little could be done to study specificity. On the other hand reasonably high agglutination titers were found in antisera from tolerant

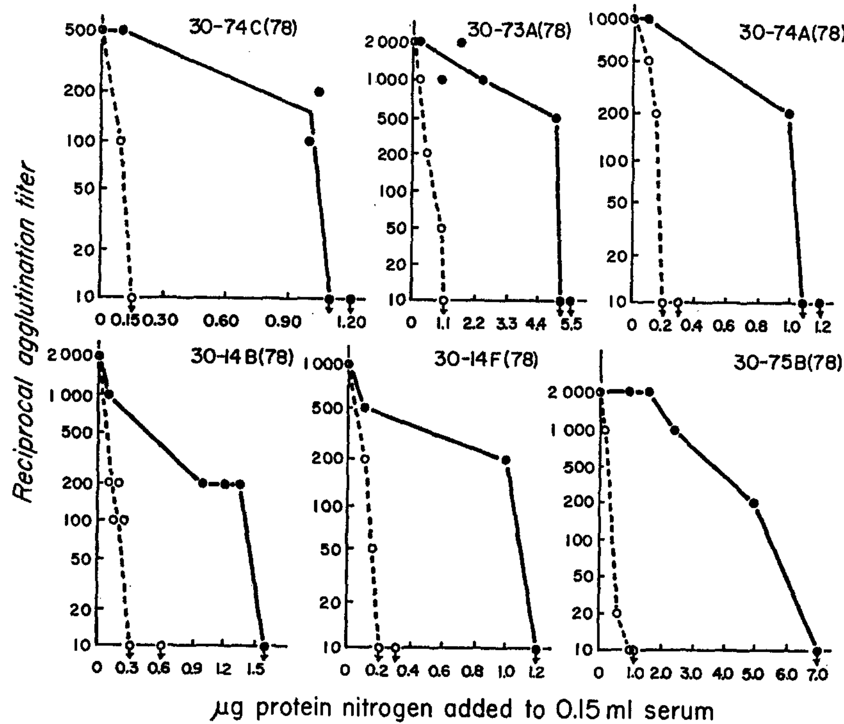

FIG. 10. The specificity of antibody to protein determinants formed by animals *injected* with HA at birth in response to immunization with HA-D_8. Sera were obtained from animals injected with HA at birth and given six injections of HA-D_8 (0.7 mg N each) between day 44 and day 73 after birth. Tanned sheep erythrocytes sensitized with HA were agglutinated by 8/58 sera. The agglutinating capacity of these antisera was inhibited by prior addition of HA-D_8 and HA. Number above each graph indicates designation of rabbits, number in brackets gives the age, in days, of the animal from which the serum was obtained. ----○----, inhibition by HA-D_8; and ——●——, inhibition by HA.

animals immunized with HA-D_8, and we have examined the specificity of this antibody. We employed agglutination-inhibition tests with tanned cells sensitized with HA, and began by examining the antibody response of control animals which had *not* been injected with HA at birth. The agglutination of this antibody was either more efficiently inhibited by HA-D_8 than by HA or was equally efficiently inhibited by the two antigens (Fig. 9). The control

animals immunized with HA-D_8 had therefore made antibodies to two types of determinants. On the basis of this variability it may be concluded that some antibodies were directed against sites which must have similar conformation on HA and HA-D_8 and the other adapted to determinants which had a different configuration on HA and HA-D_8. The relative quantities of these two antibodies were different in the sera of different animals. Similar tests showed that HA-D_8 antibody of tolerant animals was always more readily inhibited by HA-D_8 than by HA (Fig. 10). It would thus appear that the antibody made by

TABLE IV

Effect of Mercaptoethanol on the Agglutinating Capacity of Antibody from Tolerant and Control Rabbits

Control animals were not injected at birth. Animals were rendered tolerant by injection of 4 × 5 mg HA at birth. Animals were given in later life (day 44 to day 73) two courses of injections of HA-D_8 or HA-D_{35}, and were bled 5 days after the last injection of each course. Agglutinations were carried out with tanned red cells sensitized with the immunizing antigen in the absence or presence of (0.1 M) 2-mercaptoethanol.

Designation of animals	Antigen given in later life	Fraction of antisera sensitive to 2-mercaptoethanol*	
		Bleedings taken after	
		1 course	2 courses
Control	HA-D_8	6/6	0/11
	HA-D_{35}	9/9	0/11
Tolerant	HA-D_8	2/2‡	4/10
	HA-D_{35}	0/0	9/21

* This fraction gives the number of sera of which the reciprocal agglutination titer was reduced to less than 10 by mercaptoethanol treatment, divided by the number of sera in which antibody was detectable.

‡ Antibody made by these two animals was insensitive to 2-mercaptoethanol after two courses of injections.

tolerant animals was always better adapted to a determinant on the immunizing antigen and combined relatively inefficiently with a determinant on the tolerance-inducing protein. It is therefore clear that HA reactivity of antisera from tolerant animals was due to antibodies which were directed against determinants of the azoproteins.

Susceptibility of Antibody to Treatment with 2-Mercaptoethanol.—The agglutinating capacity of antibody obtained after the first course of immunization could be completely inhibited by pretreatment with mercaptoethanol, whether the antibody was obtained from controls or from tolerant animals (Table IV). The agglutinating capacity of sera obtained from control animals after two courses of injections could not be inhibited by pretreatment with

mercaptoethanol, whereas sera of 42% (13/31) of responding tolerant animals could be completely inhibited.

Hereditary Factors Controlling Antibody Response of Tolerant Animals.—We have seen that the number of antibody responders among tolerant animals depended on the nature of the protein determinants, and increased as the

TABLE V

Immune Responses of Tolerant Offspring from Random Matings and from Matings of Responding Tolerant Animals

One group of rabbits was obtained by mating between unselected animals, another group was obtained by the mating of tolerant parents which had responded to HA-D_{35}. The titers of the responding parents were measured before mating and were found to be less than 1/10. Litters were born 120–290 days after the last injection of the parents. All newborn animals were injected with HA(4×0.7 mg N). On day 44, 46, 48, 69, 71, and 73 after birth, animals were injected with HA-D_{35} (0.7 mg N). Responding animals were recognized by agglutination tests carried out with sera taken on day 54, 59, 78, and 84 after birth. Tanned cells coated with HA-D_{35} were employed in these tests.

Class of animal population	Fraction of responders*	
	Individual litters‡	Entire population§
Unselected	1/5; 0/3; 1/2; 0/3; 2/3; 3/3; 0/1; 1/3; 1/2; 1/1; 1/2; 0/2; 0/3; 3/4	14/37
Selected‖	5/6; 4/4; 4/4	13/14

* Expressed as number of responding animals in a single litter divided by the total number of animals in that litter.

‡ The incidence of responding litters in the two populations is different. The probability of identity in the proportions of litters in which more than ½ of the animals have responded is $P = 0.044$.

§ Probability that the fraction of responders in the two populations is identical, $0.005 < P < 0.01$.

‖ Offspring from tolerant animals which had made antibody in response to HA-D_{35}.

proportion of configurationally altered determinants was increased. This however left the problem unresolved as to why one animal responded and another did not.

A comparison of individual litters showed that in some litters none of the offspring made antibody whereas all of the offspring in other litters made antibody. To test whether there was a genetic factor involved in this variation, tolerant animals which responded to HA-D_{35} were mated. The offspring of such matings were rendered tolerant to HA and immunized with HA-D_{35}. The incidence of responders among these animals was higher than that of unselected animals (Table V, $0.005 < P < 0.01$). Next, the frequency of responding litters in the selected and unselected groups was compared. A responding litter was

regarded as one in which ½ or more animals have responded. In the unselected group, 6/12, and in the selected group, 3/3 were responding litters (Table V). The difference in the frequency of responding litters in selected and unselected groups was found to be significant at the 5% level ($P = 0.044$).

DISCUSSION

This investigation was carried out to analyze some of the variables which determined the specificity of antibody and the incidence of antibody responders among tolerant animals. Our discussion will consider the specificity of various antibody fractions which resulted from immunization of animals which had acquired immunological tolerance to HA and which had been immunized with two different cross-reacting antigens: HA-D_8 and HA-D_{35}.

Evidence for the presence of altered protein determinants in HA-D_8 and HA-D_{35} was obtained from agglutination-inhibition tests with HA-D_8 antibody. In some of these tests tanned erythrocytes sensitized with HA-D_{35} were employed; the agglutination of these tanned erythrocytes was equally effectively inhibited by the same nitrogen concentration of HA-D_8 as of HA-D_{35}. It thus appeared that inhibition was due to determinants shared by HA-D_8 and HA-D_{35}. If the inhibition was due to azo determinants, one would expect that the greater the concentration of these determinants, the greater would be the degree of inhibition, so that a given nitrogen concentration of HA-D_{35} would cause a greater decrease in agglutination titer than the same nitrogen concentration of HA-D_8. Thus our test did not detect an antibody fraction which was primarily directed against the azo group. Furthermore, these shared determinants could not be native protein structures since native HA reacts with this antibody but does not completely inhibit its agglutinating capacity (Fig. 6). Thus it is reasonable to conclude that the shared determinants revealed by this test are configurationally altered protein determinants. In addition to these shared determinants, there are also configurationally altered determinants which are not shared by HA-D_8 and HA-D_{35}. This was deduced from observations made in agglutination-inhibition tests which were carried out with HA-D_8 antisera and in which tanned cells sensitized with HA-D_8 were employed. It was found that the agglutination reaction could be completely inhibited by HA-D_8 and by HA-D_{25}, but that inhibition (per μg N of protein) by HA-D_8 was much more effective than by HA-D_{35} (Fig. 6, upper part). Thus the inhibitory capacity of the two derivatives was not proportional to their azo content, so that the antibody could not have been directed to the azo determinants alone. Since the antibody was better adapted to determinants on HA-D_8 than to determinants on HA-D_{35}, it would appear that the antibody was directed against conformationally altered determinants which did not have the same structure in the two derivatives. The above evidence indicates that configura-

tionally altered determinants induce antibodies and that there are differences in the conformationally altered determinants of HA-D_8 and HA-D_{35}.

We can now examine the specificity of the antibody response of tolerant animals in terms of antibody fractions which are present in tolerant animals immunized with HA-D_8 and in those immunized with HA-D_{35}. We can distinguish antibody fractions which are present in both these groups of animals, and other antibody fractions which are formed only by animals immunized with one of the two azo derivatives of HA. In this context, "shared" and "unshared" fractions were defined operationally on the basis of agglutination-inhibition test. In all cases sera from tolerant animals were compared in terms of the capacity of azo derivatives and native HA to inhibit the agglutination of tanned erythrocytes sensitized with HA-D_8 and HA-D_{35}. "Shared" fractions were characterized by identical reactions when a given set of reactants was employed; "unshared" fractions were identified by qualitative and quantitative differences in the agglutination-inhibition reaction. The presence of a "shared" antibody fraction in the sera of tolerant animals, immunized with different azo derivatives, was revealed by agglutination-inhibition tests with tanned cells sensitized with HA-D_8 and with HA and RS-D as inhibitors (Fig. 8). This "shared" fraction was directed against azo determinants since it could be inhibited by RS-D. An antibody fraction which was "not shared" was detected when the agglutination of tanned cells sensitized with HA-D_{35} was inhibited by HA-D_8 and HA-D_{35}. In these tests, antibody from tolerant animals immunized with HA-D_{35} was more effectively inhibited by HA-D_{35} than HA-D_8 (Fig. 7, upper right), whereas antibody from tolerant animals to HA-D_8 was equally effectively inhibited by the two derivatives (Fig. 6, lower right). We have discussed part of this experiment in connection with an analysis of determinants, and have shown that this antibody fraction which is "not shared" is directed against conformationally altered groups (Fig. 6). On this basis, the difference in the properties of antibody to conformationally altered groups from animals immunized with HA-D_8 and HA-D_{35} can now be interpreted. Clearly, this type of antibody, formed by tolerant animals immunized with HA-D_8, is directed to determinants "shared" by both the lightly and the heavily diazotized antigens. On the other hand, the antibody elicited by HA-D_{35} is at least partly directed to determinants which have undergone a more profound conformational alteration in HA-D_{35} than in HA-D_8. These differences in specificity can also be seen in the reactivity of these antibodies with native HA. Antibody to HA-D_8 may be expected to react more strongly with native HA than would antibody to the more highly altered protein determinants of HA-D_{35}. Indeed, antibody from tolerant animals immunized with HA-D_{35} gave very low titers with tanned cells sensitized with HA, whereas antibody to HA-D_8 gave substantially higher agglutination titers (Table III). This difference in reactivity

was also demonstrable in agglutination-inhibition tests with tanned cells sensitized with the immunizing antigen. Antibody to HA-D_8 could be partially inhibited by quantities of native HA which did not reduce the agglutinating capacity of antibody to HA-D_{35}. Thus it may be concluded that antisera of tolerant animals contain antibody to conformationally altered groups and that the reactivity with native HA can be attributed, at least in part, to antibody elicited by and adapted to determinants which are not identical with native protein determinants. Such reactivity with HA, as has so far been described, was the result of cross-reactivity with antibodies directed against conformationally altered protein determinants. This conclusion was further supported by agglutination-inhibition tests in which tanned cells coated with HA were employed. In this system only those antibodies which are adapted to protein determinants can participate in the reaction. Such tests showed that antibody from tolerant animals immunized with HA-D_8 could be completely inhibited by HA-D_8 and by HA; and that the inhibitory capacity of HA-D_8 was always greater than that of HA (Fig. 10). Thus antibody formed in tolerant animals capable of reacting with HA was better adapted to determinants present on HA-D_8 than on native HA. This antibody was therefore *not* directed against tolerance-inducing determinants, but against conformationally altered protein determinants or against determinants which consist of amino acids and the azo groups.

We have seen that the specificity of the antibody response of tolerant animals depends on the degree of modification of the immunizing antigen. Differences in specificity may be related to the higher incidence of antibody responders among animals injected with HA-D_{35}. The lightly and highly modified compounds differ in the degree of conformational changes, and in the number of protein determinants which are not identical with those of native HA. Thus a greater number of different determinants are potentially capable of inducing antibody formation in tolerant animals injected with $HA(Ox)_{38}$ and HA-D_{35} than in animals injected with $HA(Ox)_8$ or HA-D_8 (16). As a consequence, the probability must be greater that antibody will be induced by $HA(Ox)_{38}$ and HA-D_{35} than that it will be induced by $HA(Ox)_8$ and HA-D_8, and this was, in fact, found to be the case (Table II).

We can similarly account for the relatively low titer in the sera of animals immunized with HA-D_{35} in assays with tanned cells sensitized with HA. The highly diazotized compound contains a relatively small number of determinants which are structurally so similar to native ones that antibody elicited by them can react with corresponding native HA determinants. Consequently, whereas the greater degree of modification gives rise to a higher incidence of responders it elicits antibody which has very low cross-reactivity with HA.

We have now seen that the incidence of antibody responders and the relative titers of antibody can be related to the degree of modification of the immunizing

antigen. However, whether we use lightly diazotized or highly diazotized derivatives of HA, the larger proportion of tolerant animals remains unresponsive. Thus the question arose as to what factor determined whether a given animal made antibody or failed to do so. It would appear that this decision involves genetic control. The responsiveness of normal animals to antigens, which have a small number of determinants, is probably inherited under the control of a single gene (Levine, Ojeda and Benacerraf, reference 22; Benacerraf, reference 23; Pinchuk and Maurer, references 24 and 25). The inheritance of the responsiveness to complex antigens is almost certainly multigenic (Scheibel, reference 26; Sang and Sobey, reference 27; Sobey, reference 28). It will be interesting to explore this aspect in animals which are tolerant to the carrier protein and in which many or most of the determinants of a complex antigen have lost their capacity to induce antibody formation. The cellular level at which genetic control is exercised must remain a problem for future exploration.

SUMMARY

Animals were rendered tolerant to human albumin and were then immunized with azo derivatives of human albumin which differed in the number of hapten groups per molecule and in the extent of conformational change. The incidence and specificity of the resulting antibody response was studied and the presence of antibody to azo groups and to conformationally altered protein determinants was demonstrated. Reactivity with the tolerance-inducing antigen was shown to be due to antibodies directed against conformationally altered protein determinants. The difference in the response of tolerant animals to hapten-poor and hapten-rich derivatives was attributed to the extent of conformational alteration. A genetic factor appeared to be implicated in the capacity of tolerant animals to respond to an antigen which cross-reacts with tolerance-inducing macromolecules.

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