THE SPECIFICITY OF ACQUIRED IMMUNOLOGICAL TOLERANCE TO AZO PROTEINS

B. CINADER AND J. H. PEARCE*

A.R.C. Institute of Animal Physiology, Babraham, Cambridge

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EARLIER work on the specificity of acquired immunological tolerance to human albumin (H.A.) has shown that a majority of rabbits unable to form antibody to H.A. are also unable to form antibody to azo human albumin (D.H.A.). Those rabbits which did respond to D.H.A. formed antibody primarily adapted to the modified portions of the protein, but nevertheless capable of reacting with H.A., though with a remarkably low avidity (Cinader and Dubert, 1955).

Serum proteins of mammals cross-react with one another and are thus structurally similar. Acquired immunological tolerance may consequently be more readily induced by foreign serum proteins than by antigens of a structure which is radically different from that of the naturally circulating proteins of the injected new-born animal. For this reason alone the possibility of acquired immunological tolerance to D.H.A. deserved attention ; the first part of this paper is concerned with the effect of injections of D.H.A. at birth on the subsequent response to D.H.A.

The second part of this investigation deals with the effect of the injection of one azo protein on the subsequent response to another azo protein, the diazo groups being the same. We were thus examining the cross-reaction of acquired immunological tolerance to modified proteins. This problem arose not only in the context of studies on acquired tolerance but also in that of antibodies against enzymes (Cinader, 1957*a*). It was therefore of some interest to examine whether the injection of azo-rabbit serum into new-born rabbits would prevent these rabbits from responding during adult life to a heterologous antigen coupled with the same diazonium salt and if the rabbits responded, whether the resulting antibody would be adapted exclusively to the protein moiety of the antigen or to both the protein and the synthetic group.

Several reports have stressed the absence of haem specificity in antibodies to haemoglobin and catalase. This could be ascribed to acquired immunological tolerance to haem groups on the assumption that the same mechanism is responsible for acquired immunological tolerance and for the prevention or limitation of the synthesis of auto-antibodies. It is clear that a chemically modified protein will not necessarily represent an adequate model system. To choose an adequate model, information must be accumulated on the nature of cross reactivity in acquired immunological tolerance. In this context the size of the chemical grouping and nature and stability of its link to the protein may be the decisive feature. From neither of these tentative points of view does an azo protein resemble a haemoglobin. The following experiment can thus only represent a first explora-

* Present address : Microbiological Research Establishment, Porton, Wiltshire.

tion in the direction indicated. A specific example has been quoted in the haem proteins to illustrate reasons for the continued examination of a wider problem, that of the nature of cross reactivity in immunological tolerance.

The following systems of proteins coupled with the diazonium salt of *p*-amino benzene sulphonic acid were investigated :

1. Injection immediately after birth : azo human albumin (D.H.A.) ; injection in adult life : azo ribonuclease (D.Rn (18)).
2. Injection immediately after birth : azo rabbit serum (D.R.S.) ; injection in adult life : azo human albumin (D.H.A.).
3. Injection immediately after birth : azo ribonuclease (D.Rn) ; injection in adult life : azo human albumin (D.H.A.).

The antibodies in rabbits from the above three groups were examined for their protein and for their azo specificity (including such new specificity as may be imposed on the protein structure itself in consequence of diazotisation) ; only in the first of the three groups was the response to the antigen injected at birth investigated by the response in adult life to the same antigen. In group 3 this was frustrated by the poor antigenicity of D.Rn and Rn in adult white Himalayan rabbits ; in group 2 it was not attempted.

MATERIALS AND METHODS

Protein antigens

The immunological properties of the native proteins used in this investigation have been described in earlier publications (human albumin (H.A.) : Cinader and Dubert, 1955 ; bovine ribonuclease (Rn) : Cinader, Rondle and Pearce, 1955 ; Cinader and Pearce, 1956). The preparation and properties of the azo human albumin (D.H.A.) used in the following experiments has been reported by Cinader and Dubert (1955, 1956).

Ribonuclease and rabbit sera were coupled with diazonium salt in an ice water bath. The diazonium salt of sulphanilic acid, cooled to 0°, was added drop by drop to the protein solutions and the pH was kept between 8·0–9·0 by the addition of 1 N NaOH.

Sulphonic acid containing molecules labelled with S^{35} was used to measure the number of chemical groups introduced into Rn. The radioactivity in thoroughly dialysed preparations of the chemically modified proteins was compared with that of the solution of sulphonic acid used in the preparation of the diazonium salt.

Azo ribonuclease (D.Rn).—D.Rn was prepared from crystallised bovine ribonuclease (Armour). The protein was coupled with the diazonium compound of benzene-*p*-sulphonic acid.

Two products were prepared which differed in the degree of coupling.

1. *D.Rn* (7).—The concentration of the diazonium salt was adjusted so that 0·16 mg. sulphanilic acid were added for each mg. of ribonuclease. The ratio between the S contained in the azo groups and the protein nitrogen was 0·107.

2. *D.Rn* (18).—The concentration of the diazonium salt was adjusted so that 0·48 mg. sulphanilic acid were added for each mg. of ribonuclease. The ratio between the S contained in the azo groups and the protein nitrogen was 0·282.

The modification of these two preparations of azo ribonuclease can be judged from their reaction with ribonuclease antibody. While D.Rn (7) precipitates nearly as much of this antibody as does Rn, D.Rn (18) precipitates only a small fraction of ribonuclease antibody (Fig. 1).

Azo-rabbit serum (*D.R.S.*).—The concentration of the diazonium salt was adjusted so that 0·50 mg. sulphanilic acid were added per mg. of protein.

Reagents

Reagents used in the estimation of nitrogen by Microkjeldahl were M.A.R. grade. Other reagents were A.R. grade and were dissolved in glass distilled water.

Polyvidone.—Polyvinyl pyrrolidone (free of disinfectants) was obtained from May & Baker Ltd. as a 25 per cent solution.

Polyvidone saline.—Polyvinyl pyrrolidone (0·35 per cent) in 0·15 M-NaCl.

Sulphanilic acid (S^{35}).—Radiochemical purity was examined by isotope dilution analysis and was found to be 99 per cent compared with laboratory reagent sulphanilic acid. Paper chromatography in a mixture of ethanol, ammonia and water (80 : 4 : 16) showed one spot of $R_F = 0·5$ on autoradiography and did not show a spot corresponding to sulphate ion ($R_F = 0·1$).

Immunological methods

Strain of rabbits.—The rabbits used in these experiments were white Himalayan rabbits obtained from the National Institute for Medical Research, except 87A, C, D, E, H, 88E,

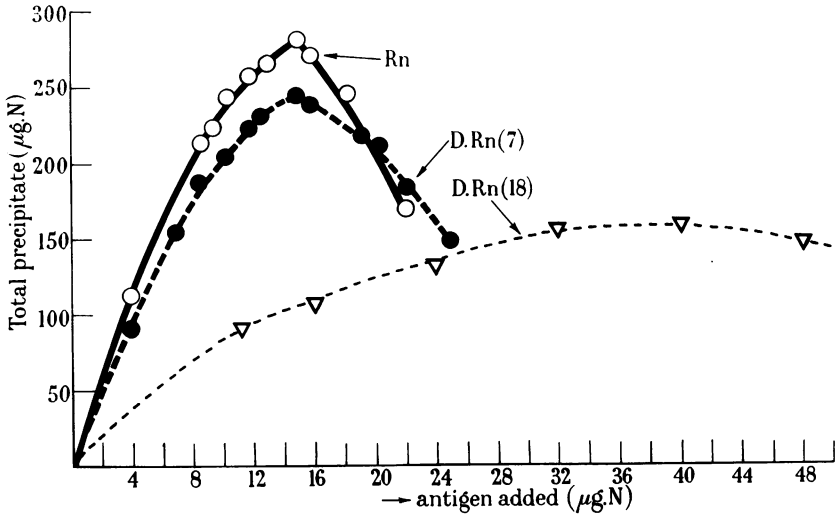

FIG. 1.—Precipitation of Rn, D.Rn (7) and D.Rn (18) by ribonuclease antiserum (Pool 3).

▽—▽ quantitative precipitin curve for D.Rn (18) and Rn antiserum.

○——○ quantitative precipitin curve for Rn and Rn antiserum.

●- - -● quantitative precipitin curve for D.Rn (7) and Rn antiserum.

110, 111, 112 and 114 which were wild type rabbits and 63 M, A, C, E which were red New Zealand rabbits.

Designation of rabbits.—Individual rabbits were distinguished by a number shared by all the individuals of the same litter and a letter (A to H); the mothers were described by the number of their litter and the letter M.

Injections of new-born rabbits.—New-born rabbits were injected intraperitoneally with protein solutions dissolved in saline (0·15 M-NaCl); the first injection was given within 12 hr. of birth.

Injections of adult rabbits.—Injections were made into the marginal vein of the ear of protein dissolved in saline. A course of injections consisted usually of three injections administered at intervals of 48 hr. Rabbits were bled 11 days after the first course and 5 (sometimes also 11) days after the last injection of the second and subsequent courses.

Agglutination (see Boyden, 1951; Borduas and Grabar, 1953).—Sheep erythrocytes were washed three times with saline, suspended in phosphate-saline buffer (pH 7·2) to give a 2·5 per cent suspension and were then mixed with an equal volume of tannic acid (1/20,000) in saline; after 10 min. the tanned erythrocytes were centrifuged, washed with phosphate buffer at pH 7·2 and suspended in saline. 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 coal gas for 2 min., the optical density of the resulting solution was 0·3 (EEL Colorimeter, Ilford filter No. 624) in a tube 1·5 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 and sensitised erythrocytes were centrifuged and washed three times with polyvidone saline solution.

The concentration of these tanned and sensitised cells was adjusted as before.

Sera were heated in stoppered tubes at 56° for 60 min. Natural sheep antibodies were absorbed for 1 hr. with 0·1 ml. of washed centrifuged sheep erythrocytes per ml. of serum and the erythrocytes were then removed by centrifuging. Sera were diluted in polyvidone-saline, and to each of the serum dilutions was added one drop of the suspension of tanned and sensitised sheep erythrocytes from a pipette calibrated to deliver 0·05 ml. per drop. We were unable to obtain any results with cells which we attempted to sensitise with D.Rn (18), we have therefore assayed sera obtained after immunisation with either D.Rn (7) or D.Rn (18) with tanned erythrocytes sensitised with D.Rn (7).

Examination of the specificity of antibodies by agglutination inhibition.—This method has been applied in earlier studies of acquired tolerance (Cinader and Dubert, 1955) and in the comparison of ribonuclease A and B (Cinader and Pearce, 1956). Tanned sheep erythrocytes were sensitised with azo proteins and were agglutinated with antibody formed in response to these azo proteins. The agglutination titre was reduced if the native protein was allowed to react with the immune serum before tanned and sensitised cells were added.

Different quantities of inhibitors dissolved in saline, 0·15 ml. of immune serum, and a quantity of polyvidone to give a final concentration of 0·35 per cent, were made up with saline to give a final volume of 1·5 ml. The mixtures were incubated for 2 hr. at 37°, the kept at 2 ± 1° overnight and were finally centrifuged. The agglutination titres of the immune sera were subsequently examined and compared with those of sera to which antigen had not been added. The azo proteins used in these experiments were D.H.A. and D.Rn and the antigens used for inhibition were principally H.A. and Rn.

Nitrogen assays of antigen-antibody precipitates.—Varying amounts of antigen were added to constant quantities of antibody and were immediately mixed to reduce the Danysz effect (Danysz, 1902 ; see also Cinader, 1957*b*). Mixtures were adjusted to constant volume with phosphate saline buffer (pH 7·5). The mixtures were kept at 37° for 2 hr. and then at 2° for 6 days. The precipitates were centrifuged at 4°, washed twice with 2 ml. of ice cold phosphate saline buffer and then assayed for nitrogen by the micro-Kjeldahl method.

The determination of heterologous antigen in the blood stream of rabbits.—Rabbits were given 3 intravenous injections of 10 mg. of H.A. at intervals of 48 hr. and were bled on the fifth, eleventh and eighteenth day after the last injection. The blood was allowed to clot and serum was separated.

Sera from rabbits immunised by repeated courses of intravenous injections with H.A. were pooled ; two such pools were used. The nitrogen precipitated by varying known quantities of H.A. from a constant volume of each of these pooled sera was determined, and a calibration curve was drawn by plotting antigen N added against total N precipitated.

Rabbit sera to be assayed for human albumin were added to the same quantities of pooled H.A. antisera ; the precipitated nitrogen was measured and compared with the nitrogen precipitated by known quantities of H.A. Samples of unknown antigen content were usually determined in the region of excess antibody.

The experimental procedure was adjusted so that the conditions in which the calibration curve was determined corresponded closely to those prevailing in the determination of the quantity of H.A. in the serum of injected rabbits. To this end all mixtures of antigen and antibody were adjusted by the addition of normal rabbit serum to contain finally the same amounts of rabbit serum.

All mixtures of antigen and antibody were incubated for 2 hr. at 37° and then kept for 6 days at 2°. Precipitates were isolated by centrifugation at 4°, washed twice with phosphate saline and assayed for nitrogen.

The half life of the antigen was calculated from the equation

$$\text{half life} = \frac{t}{\log n_1 - \log n_2} \times 0{\cdot}301$$

where n_1 and n_2 was the amount of human albumin found in 1 ml. of rabbit serum on the fifth and eleventh day respectively and t was the time in days (6) between the two determinations.

RESULTS

The response to injections with H.A.

It has been shown (Cinader and Dubert, 1955) that 20 injections of 1 mg. of H.A. given to new-born rabbits caused immunological tolerance. The effect of smaller numbers of injections was examined. A single injection of 5 mg. of H.A. did not seem to affect the immunological response to the same antigen given in later life in four out of five rabbits (Table I).

Rabbits (88E, 266A) injected four times with 10 mg. H.A. during the first 3 days of their life received during adult life three courses of immunisation with H.A., each consisting of three injections at intervals of 48 hr. During the first course 15 mg. and during each of the two subsequent courses 30 mg. of H.A. were administered. The agglutination titre to H.A. was measured 5 and 11 days after the last injection of each course of immunisation and was found to be less than 1/5 in every instance.

TABLE I.—*The Response to H.A. During Adult Life of Rabbits Injected Intraperitoneally with 5 mg. H.A. Immediately After Birth and of Controls*

(*a*) Intraperitoneal injections immediately after birth.

Litter	87†	63†
Individuals injected	E, H	E*, G, H*
Quantity of H.A. injected (mg.)	1×5	1×5

(*b*) Dose injected into adult rabbits.

Total mg. H.A. injected in each course (at intervals of 48 hr.)	3×5 . 3×10 . 1×20 . 3×10	3×5 . 3×10 . 3×10 . 3×10

(*c*) Agglutination titre of tanned erythrocytes sensitised with H.A.

Bleedings‡: days after birth	80	100	118	339	71	77	97	124	262
Rabbits A	<5	200	1,000	20,000	§	§	§	§	§
C	<5	<5	<5	1,000	500	500	500	500	20,000
D	2,000	§	§	§	§	§	§	§	§
E	**<5**	**200**	**2,000**	**10,000**	**100**	**<5**	§	§	§
G	§	§	§	§	**<5**	**<5**	**<5**	**<5**	**1,000**
H	**200**	§	§	§	**<5**	**<5**	**<5**	**<5**	**<5**
M	§	§	§	§	<5	<5	<5	<5	5,000

* These rabbits received an injection of 1 ml. normal rabbit serum together with the injection of H.A.

† The animals in both these litters were severely affected by coccidiosis during the 6th and 7th weeks of their life and the poor and slow antibody response may be due to this fact.

‡ Bleedings: 11 days after the last injection of the first course, and 5 days after the last injection of subsequent courses.

§ Died

Titres in heavy type are measured on sera from animals injected at birth.

Response during adult life to injections with D.H.A. of rabbits injected with D.H.A. immediately after birth

Details of the injections given to new-born rabbits and the response of adult animals to injection of D.H.A. are shown in Table II. A single injection of 5 mg. D.H.A. within the first few hours after birth did not affect the subsequent immuno-

logical response of the rabbit. Twenty post-natal injections of 1 mg. each or of 4 mg. each or four post-natal injections of 10 mg. each depressed or inhibited completely the subsequent response to D.H.A. during adult life. The observed effect differed from that found where H.A. was injected at birth and subsequently during the adult life of the rabbit. While the injection with H.A. suppressed a later response to this antigen either completely or not at all, the injection with D.H.A. at birth allowed in some animals a low residual response to injections with D.H.A. when the animal was injected after the second month of its life. This response which could only be demonstrated by agglutination of tanned and sensitised erythrocytes, was very weak, and differed from normal immunological responses by a decrease of antibody titre in response to successive courses of injections. The extent of this response and the degree of its decline after successive injections was variable even within a group of animals from the same litter injected in the same manner. This was most noticeable when the response of rabbits 146A, 146B and 146C was compared (Table II).

It seemed possible to attribute these differences observed in parallel experiments with H.A. and D.H.A. to the different rate of elimination of these two antigens (Cinader and Dubert, 1956). H.A. is relatively slowly eliminated from the blood stream of normal rabbits and might therefore mask the release of small quantities of antibody. D.H.A. is very rapidly eliminated and the presence of small quantities of antibody in the circulation might, therefore, be more readily demonstrable. To test this possibility the elimination of H.A. from the blood stream of the rabbits injected at birth and during adult life with D.H.A. was examined (Table III). The elimination of H.A. was also studied in three rabbits (372A, B, C) which had been injected in the same manner as 146A, B, C with four doses of 10 mg. D.H.A. during the first 3 days of life, but which had not been injected with D.H.A. subsequently. In addition, the elimination of H.A. was measured in rabbits which had been injected with H.A. at birth.

Rabbits injected at birth with D.H.A. which had subsequently shown a more or less transient response to D.H.A. were found to eliminate H.A. more rapidly from their blood stream than other rabbits in the same group or than rabbits injected with H.A. at birth. The elimination of H.A. reflected the initial response of the rabbits. The three rabbits from litter 146 had shown a gradation of response to D.H.A.; 146A had responded with the highest titre and in every one of four courses of injections, 146B had formed antibody demonstrable by agglutination after the first and second course of injections and 146C only after the first course (Table II). In the subsequent studies of elimination circulating antigen was found in 146A on the 5th day after the last injection but not subsequently, in 146B on the 5th and 11th but not on the 18th day, and in 146C on the 5th, 11th and 18th day. Only in the serum of rabbit 146A was antibody detected (Table IV).

The half life of human albumin determined in 146C agreed with that determined in rabbits which had been injected with H.A. at birth. The other rabbits which had been injected with D.H.A. at birth eliminated H.A. at the same rate as rabbits injected with H.A. at birth, except 264D which had previously shown a relatively strong response to D.H.A. Elimination studies with H.A. have thus reflected the response to D.H.A. (Tables II and III); only a small proportion of the responding animals would have been recognised as such if the antibody response to H.A. (rather than elimination) had been determined. It remained questionable whether injection with H.A., not preceded by immunisation with

TABLE II.—*Antibody Response to Injections of D.H.A. of Rabbits Injected with D.H.A. at Birth and of Controls*

(*a*) Intraperitoneal injections immediately after birth.

Litter	102	146	263	264	372
Individuals injected	E, F, G	A, B, C	C, E	C, D	A, B, C
Total quantity of D.H.A. (mg.) injected (at intervals of 24 hr.)	1×5	4×10	20×4	20×1	4×10

(*b*) Dose injected into adult rabbits.

Total mg. D.H.A. injected in each course (at intervals of 48 hr.)	3×5 . 3×10 . 3×10	3×5 . 3×10 . 3×10 . 3×10	3×5 . 3×10 . 3×10	3×5 . 3×10 . 3×10	3×10‖ . 3×10

(*c*) Agglutination titre of tanned erythrocytes sensitised with D.H.A.

Bleedings‡ : days after birth.		77	97	124	77	97	128	305	77	97	138	77	97	128	186	205
Rabbits	A	*	*	*	**200**	**100**	**100**	**100**	§	§	§	§	§	§	**<5**	**<5**
	B	*	*	*	**100**	**50**	**<5**	**<5**	§	§	§	§	§	§	**<5**	**<5**
	C	*	*	*	**100**	**<5**	**<5**	**<5**	**<5**	**<5**	**<5**	**50?**	**<5**	**<5**	**<5**	**<5**
	D	§	§	§	§	§	§	§	§	§	§	**<5**	**200**	**5**	50,000	50,000
	E	**1,000**	**10,000**	**20,000**	§	§	§	§	**10**	**<5**	**<5**	§	§	§	5,000	10,000
	F	**200**	**20,000**	**50,000**	†	†	†	500	—	—	—	§	§	§	100?	5,000
	G	**200**	**20,000**	**50,000**	2,000	20,000	50,000	20,000	§	§	§	§	§	§	§	§
	M	*	*	*	2,000	50,000	100,000	50,000	500	20,000	50,000	§	§	§	20,000	50,000
	112	2,000	50,000	20,000												
	114	2,000	20,000	20,000												

* See Table V.
† Not injected.
‡ Bleedings : 11 days after the last injection of the first course and 5 days after the last injection of subsequent courses.
§ Not available.
‖ Injected with H.A. before injections with D.H.A. were given (see Table III).
Titres shown in heavy type have been measured in sera of rabbits injected at birth with D.H.A.
? Indicates the presence of a prezone.

TABLE III.—*The Elimination of H.A. from the Blood Stream of Rabbits Injected with H.A. at Birth, and from Rabbits Injected with D.H.A. at Birth*

Each adult rabbit received 3 i.v. injections with H.A. at intervals of 48 hr. and was bled 5 days, 11 days and sometimes 18 days after the last injection.

Antigen injected immediately after birth	Litter	Individual	Day of last injection	Day of bleeding	H.A. in circulation μg./2 ml.	Half life of H.A.		
						Individual values (measured between 5th and 11th day)	Mean	Standard error
None		M		423	—	—	—	—
				429	—			
				436	—			
D.H.A.	146	A	418	423	13·25	—	—	—
				429	—			
				436	—			
		B		423	5·0	3·5	—	—
				429	1·5			
				436	—			
		C		423	18·9	4·8	—	—
				429	8·0			
				436	3·25			
	264	D	196	201	23·25	—	—	—
				207	—			
		C		201	27·5, 30·0	5·7, 6·0	5·60	0·25
				207	13·25, 15·0			
	263	C	256	261	31·0	5·6		
				267	14·75			
				274	5·0			
		E	207	212	24·25	5·9		
				218	12·0			
	372	A	99	104	17·5	5·0		
				110	7·6			
				117	3·8			
		B		104	18·75	5·3		
				110	8·5			
				117	4·25			
		C		104	15·25	5·3		
				110	7·0			
				117	2·75			
H.A.	266	A	97	102	10·5	4·0	5·12	0·22
				108	3·75			
			128	133	15·3	5·7		
				139	7·4			
			197	202	29·5, 30·0	6·0, 5·3		
				208	14·75, 13·75			
	88	E	208	213	9·9	5·0		
				218	5·0			
	693	C*	722	727	8·5	5·5		
				733	4·0			
	712	C*	657	662	8·5	4·7		
				668	3·5			

* For details of the injections given at birth see Cinader and Dubert (1955).

TABLE IV.—*The Response to H.A. of Animals Injected with D.H.A. at Birth and of Controls* (cf. Table III)

(*a*) Dose.

Litter (individual rabbits)	146 (**A, B, C,** F, G, M)
Injection with H.A., days after birth	414
	416
	418
Total mg. H.A. injected	3 × 10

(*b*) Agglutination titre of tanned erythrocytes sensitised with H.A.

Bleedings: days after birth		423	429	436
Rabbits	**M**	20,000	20,000	20,000
	A	<5	**100**	**50**
	B	<5	<5	<5
	C	<5	<5	<5
	F	10,000	5,000	1,000
	G	5,000	2,000	1,000

Titres and letters in heavy type refer to rabbits injected with D.H.A. immediately after birth.

D.H.A., would have revealed a more rapid elimination of antigen. However, the rabbits (372 A, B, C) which had been injected with D.H.A. at birth but had not been subsequently injected with D.H.A., eliminated H.A. in a manner indistinguishable from that of animals injected after birth with an effective dose of H.A. Rabbits from litter 372 were subsequently injected with D.H.A. and did not form antibody to it (Table II). The experiment with litter 372 had thus failed to support but had not disproved the proposition that the difference in the response to H.A. and D.H.A. was due to the difference in the elimination of the two antigens from the circulation.

We have so far seen that the antibody response of rabbits injected with D.H.A. immediately after birth is completely or almost completely inhibited, that there is a strong variability in the extent of the feeble residual response within animals from different litters and that though the residual response declines during successive courses of immunisation it remains demonstrable by the rate of elimination of H.A. from the blood stream. It has been impossible to support the view that this difference between rabbits injected with H.A. and with D.H.A. at birth is due to the different rate of elimination of this antigen when administered during adult life. The rapid elimination of D.H.A. by a constitutive process may allow only a relatively small portion of this antigen to reach antibody forming cells.

It is likely that the different rate of elimination of H.A. and D.H.A. observed in adult normal rabbits would also be found in new-born animals, particularly since this difference is not due to an immune response (Cinader and Dubert, 1957). If this is so, a smaller percentage of the administered dose may be reaching potential antibody-forming cells when D.H.A. rather than H.A. is injected.

Response during adult life to injections with D.H.A. of one rabbit injected with H.A. immediately after birth

A small number of rabbits with acquired immunological tolerance to H.A. respond during adult life to injections with D.H.A. by the formation of an antibody (Cinader and Dubert, 1955 ; 1956). Another animal of this type was encountered in 266A (See Table V). The elimination of H.A. from the circulation of this rabbit

was similar to that of other rabbits which had acquired tolerance to H.A. (Table III). The rabbit did not respond demonstrably to the first but did respond to the second and third course of injections with D.H.A., the titre developed after the third course being considerably higher than after the second course (Table V). The agglutination by these sera of tanned cells sensitised with D.H.A. was readily inhibited by D.H.A. but was not inhibited by H.A. (Fig. 2). So far the antibody response to D.H.A. of rabbits injected at birth with D.H.A. and H.A. has been examined. The following experiments were designed to examine the antibody response to D.H.A. in rabbits injected at birth with other azo proteins.

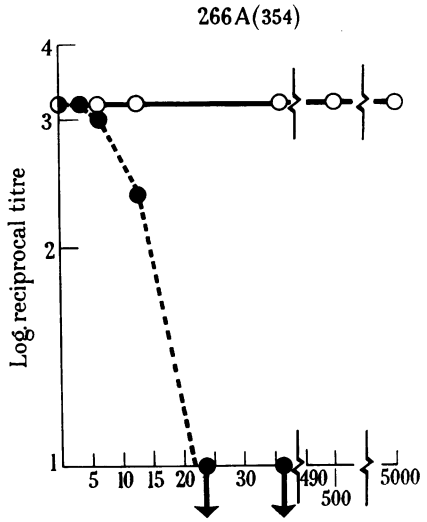

FIG. 2.—The inhibition by H.A. and D.H.A. of agglutination-erythrocytes sensitised with D.H.A. D.H.A. antiserum from a rabbit injected with H.A. at birth and later immunised with D.H.A. (Table IV).

Number in brackets gives age of the donor in days.

○——○ agglutination inhibited by H.A.

●- - -● agglutination inhibited by D.H.A.

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates μg inhibiting Antigen/0·15 ml. Antiserum.

Response during adult life to injections with D.H.A. of rabbits injected with D.R.S. or with D.Rn immediately after birth

The introduction of azo groups into H.A. brought about a weight difference of only 3 per cent between native and chemically modified protein ; the principal portion of the H.A. and D.H.A. remained essentially similar. The percentage of dissimilar antigenic determinants becomes very much higher when two different modified proteins are compared ; in this instance, the difference is due to the protein moiety and hence in 97 per cent of the molecular weight of D.H.A.

The above considerations have no quantitative but only qualitative significance ; they neglect original similarities in amino acid sequences and changes in protein configuration which may be caused by the introduction of the diazo groups.

Coupling with diazonium salt may increase the similarity of two proteins in surface structure or spatial arrangement. Nevertheless the consideration in terms of weight may give some indication of the considerable difference between the system investigated at present and that described in earlier publications.

The injections of D.Rn and D.R.S. given to new-born rabbits within the first days after birth are shown in Table V. The quantity of D.R.S. which was injected into new-born rabbits exceeded the quantity of purified proteins injected into other rabbits so that the amount of azo rabbit albumin contained in the mixture of rabbit proteins should be similar to the weight of the purified proteins.

The rabbits injected immediately after birth and their litter mates received during adult life three courses of injections with D.H.A. The response to D.H.A. of animals injected at birth with azo proteins other than D.H.A. did not differ from that of the controls (Table V). Thus a very marked difference was found between the results obtained here and those observed in the response to D.H.A. of rabbits injected immediately after birth with H.A., when the majority of the rabbits had been found to be equally unresponsive to the native and to the chemically modified protein.

It remained to examine qualitatively the response in the rabbits injected with D.Rn or D.R.S. at birth and in controls. It seemed possible that animals injected with D.Rn or D.R.S. at birth might have formed an antibody entirely adapted to the protein moiety of H.A., while animals not injected at birth would have formed antibody adapted to the azo group as well as antibody adapted to the protein determinants. Two sera differing in this way could be distinguished by inhibition with H.A. Were the antibodies exclusively adapted to H.A., inhibition with H.A. would completely abolish the reaction of tanned cells sensitised with D.H.A. This would not be the case if some of the antibody molecules were primarily adapted to the sulphanilic acid group.

A number of control animals received three courses of immunisation with D.H.A. The agglutination by the resulting D.H.A. antisera of erythrocytes sensitised with D.H.A. was completely inhibited by the prior addition of D.H.A. to the antisera but only partially inhibited by the addition of H.A. A plot of reciprocal agglutination titre as a function of the quantity of H.A. added to a D.H.A. antiserum descends steeply and finally reaches a more or less constant level (Fig. 3). For D.H.A. antisera from animals not injected at birth, the initial portion of steep descent did not reach titres of less than 1/10 (Fig. 3). An exception to this was found in the antibody from a rabbit which had been immunised with H.A. before being immunised with D.H.A. The D.H.A. antisera considered in the following comparison were obtained from rabbits which had not been injected with H.A. before being immunised with D.H.A. Some D.H.A. antisera from controls were completely inhibited by H.A. in extreme antigen excess* (2000–3000 μg./0·15 ml.) While this is encountered quite frequently after the first course of immunisation, sera obtained from controls after two to three courses of immunisation gave a residual titre even in the presence of a vast excess of antigen.

The difference between litters was tested in a one-way classification analysis of variance, comparing the variation between litters and within litters. The difference between litters was found to be more marked at higher than at lower levels of inhibiting H.A. For this reason the subsequent analysis was carried out on litter means. Differences between groups were only accepted as significant if significant differences could be demonstrated within a group of animals from

TABLE V.—*Antibody Response to Injections with D.H.A. of Controls and of Rabbits Injected at Birth with D.Rn* (7), *D.Rn* (18) *and D.R.S. and of one Rabbit* (266A) *Injected at Birth with H.A.*

Litter	102	194	266*	262	373
Individuals injected	A, B	B, C, D, E	C, E	D	A, B, C, D
Antigen injected	D.Rn (7)	D.Rn (18)	D.Rn (18)	D.R.S.	D.R.S.
Total quantity (in mg.) injected (at intervals of 24 hr.)	1×5	4×10, then 15×1	20×1	20×4	20×20

(*b*) Dose injected into adult animals.

Total wt. of D.H.A. injected (at intervals of 48 hr.)	3×10	3×10	3×5	1×10	3×10	1×10	3×5	3×10	3×10	3×5	3×10	3×10	2×5	3×10	3×10

(*c*) Agglutination titre of tanned erythrocytes sensitised with D.H.A.

Bleedings‡: days after birth		296	316	220	237	407	417	430	303	323	329	354	79	97	138	81	102	133
Rabbits	A	**200**	**20,000**	§	§	§	§	§	<5*	200*	100*	2,000*	§	§	§	**500**	**10,000**	**10,000**
	B	**2,000**	**20,000**	**1,000**	**10,000**	**20,000**	§	†	§	§	§	§	§	§	§	**500**	**20,000**	**5,000**
	C	§	§	**500**	**5,000**	**10,000**	§	†	**1,000**	**10,000**	**5,000**	**10,000**	§	§	§	**200**	**20,000**	**20,000**
	D	§	§	**5,000**	**10,000**	**5,000**	§	†	§	§	§	§	**10,000**	**50,000**	**50,000**	50	20,000	10,000
	E	§	§	**2,000**	**10,000**	**20,000**	§	†	**1,000**	**10,000**	**5,000**	**20,000**	§	§	§	500	20,000	20,000
	F	§	§	†	†	<5	100	10,000	§	§	§	§	§	§	§	§	§	§
	M	200	50,000	5,000	10,000	10,000	§	†	2,000	20,000	20,000	20,000	10,000	10,000	200,000	500	50,000	20,000
	110	1,000	20,000															
	111	1,000	20,000															

* 266A injected with H.A. at birth (4×10 mg. at intervals of 24 hr.).
† Not injected.
‡ Bleedings: 11 days after the last injection of the first course and 5 days after the last injection of each subsequent course.
§ Not available.
‖ Injected with ribonuclease at birth.
Titres shown in heavy type have been measured in sera from rabbits injected at birth with D.Rn or D.R.S.

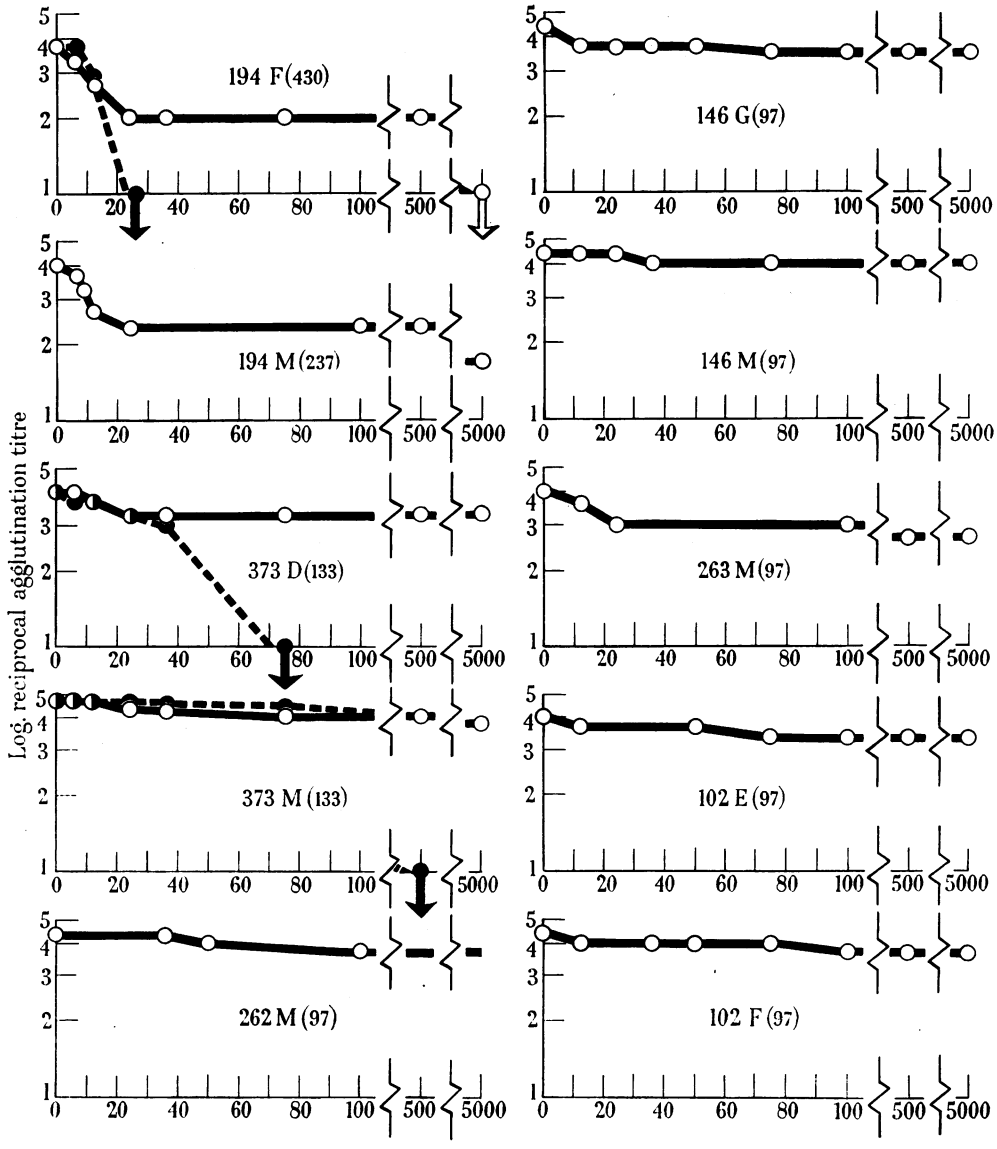

FIG. 3.—Controls.

The inhibition by H.A. (and D.H.A.) of agglutination-tanned erythrocytes sensitised with with D.H.A.

D.H.A. antisera from adult normal animals not injected with D.H.A. at birth and from rabbits 102E and 102F which received a single injection of 5 mg. D.H.A. immediately after birth, a dose which does not affect their subsequent response (see Table II). Values in brackets give the age of the donor animal, except in the case of the mothers (M) where the number in brackets refers to the days after the birth of their litter.

○——○ agglutination inhibited by H.A.

●- - -● agglutination inhibited by D.H.A.

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates μg inhibiting Antigen/0·15 ml. Antiserum.

the same litter as well as between groups of animals derived from different litters. The results of inhibition-agglutination with sera from the control group were compared with those with the D.H.A.-antisera from animals injected with

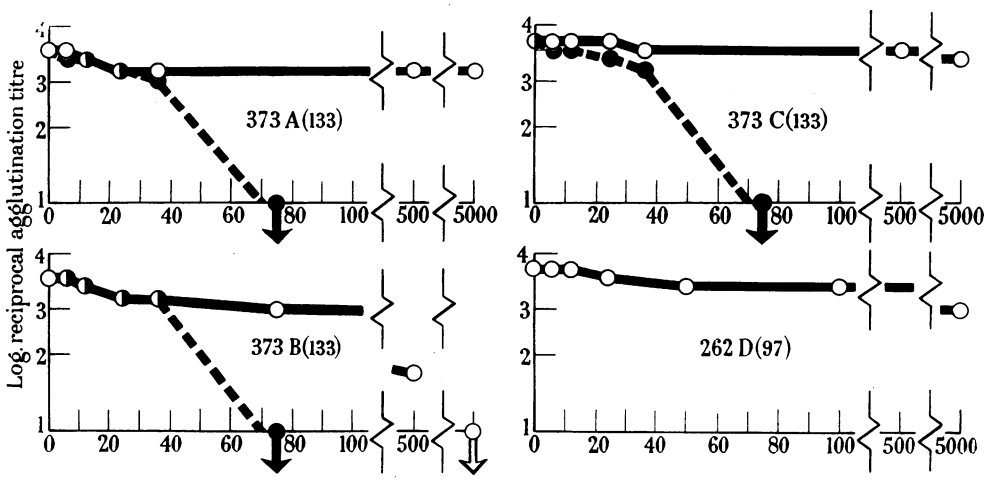

FIG. 4.—Rabbits injected with D.R.S. at birth.

The inhibition by H.A. (and D.H.A.) of agglutination-tanned erythrocytes sensitised with D.H.A.

D.H.A. antisera from adult animals injected with D.R.S. at birth (see Table V). Values in brackets give the age of the donor animal.

○——○ agglutination inhibited by H.A.

●- - -● agglutination inhibited by D.H.A.

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates μg inhibiting Antigen/0·15 ml. Antiserum.

D.R.S. at birth (Fig. 4) and with those from animals injected with D.Rn (18) at birth (Fig. 5). The variable considered was :

$$x_n = \log (t_n/t_o) \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where t_n = titre with nx μg. of inhibiting H.A. and t_o = titre without inhibiting H.A.; $x = \frac{t_o}{10{,}000}$, n = μg. protein/0·15ml. serum.

It can be seen from Table VI that the antibody from rabbits injected immediately after birth with D.R.S. did not differ from that of controls. The D.H.A. antibody from rabbits injected at birth with D.Rn (18) appeared to be more effectively inhibited by H.A. than that of controls (Fig 5). This became significant in slight antigen excess, corresponding to fairly low concentrations of inhibiting H.A. (Table VIa). The significance of this difference was also tested within a group of rabbits from the same litter (194) by computing the difference between the x_n (1) of controls and of animals injected with D.Rn (18) at birth. The regression of this difference on the logarithm of the inhibiting dose was significant on the 5 per cent level (Table VIb). If the regression of Δx_n (D.Rn-control) was

determined against 1, 2, 3, 4, 5 (representing the different values of nx), it was found to be significant at the 1 per cent level ($F = 50{\cdot}83$ on 1, 3 degrees of freedom). On the other hand, a similar comparison between rabbits injected at birth with D.R.S. and controls from litter 372 and 262 showed the regression to be not significant (Table VIb). The comparison within litters and between litter means

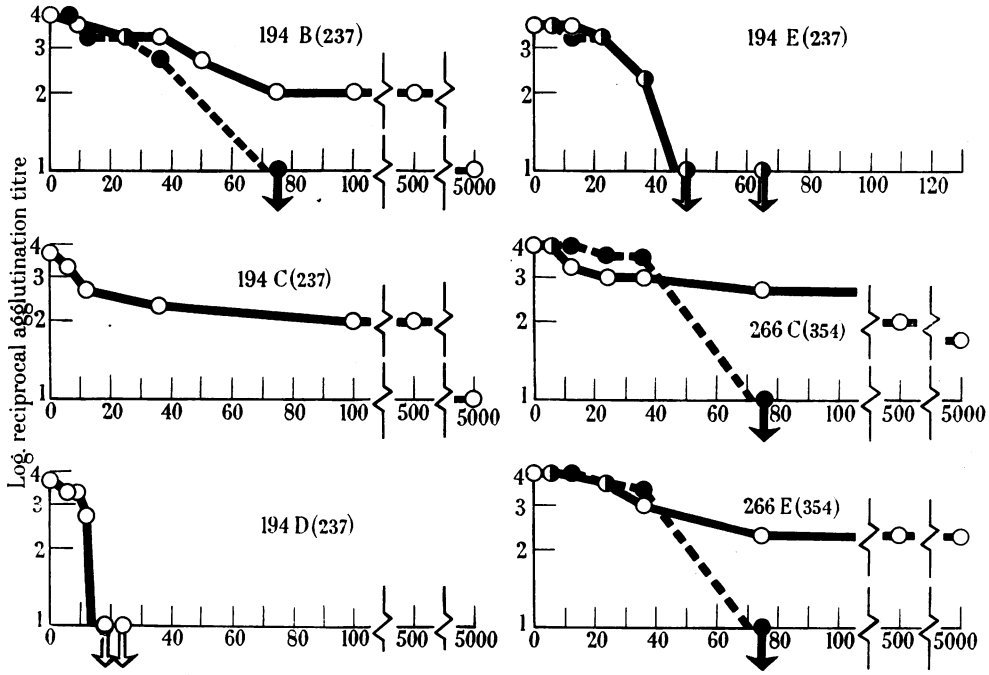

FIG. 5.—Rabbits injected with D.Rn (18) at birth.

The inhibition by H.A. (and D.H.A.) of agglutination-tanned erythrocytes sensitised with D.H.A.

D.H.A. antisera from adult animals injected with D.Rn (18) at birth (see Table V). Values in brackets give the age of the donor animal.

agglutination inhibited by H.A.

agglutination inhibited by D.H.A.

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates μg inhibiting Antigen/0·15 ml. Antiserum.

showed that D.H.A. antibody from rabbits injected immediately after birth with D.R.S. did not differ from antibody of the controls and that D.H.A. antibody from rabbits injected immediately after birth with D.Rn (18) differed from antibody of controls.

A difference between the antibodies of controls and of rabbits injected at birth could not be demonstrated by inhibition of agglutination with D.Rn or D.R.S. Inhibition of agglutination was never greater than one dilution step and was therefore within the limits of error of the method.

TABLE VI.—*The Inhibition of D.H.A. Antisera from Rabbits Injected Immediately after Birth with D.R.S., D.Rn* (18) *and from Controls (Test of Significance)*

The variation of x_n* with inhibiting dose

(*a*) Comparison of litter means.

Inhibiting dose n (μg. H.A./0·15 ml.)		10	20	40	80	500
x_n	Control	0·304	0·584	0·758	0·850	0·913
	D.R.S. at birth	0·172	0·500	0·590	0·633	0·967
	D.Rn (18) at birth	0·271	0·541	1·277	1·889	2·312
Comparison of litter means	Controls and rabbits injected with D.R.S. at birth: $t>$	0·924	0·311	0·490	0·608	0·137
	Controls and rabbits injected with D.R.S. at birth: $>P>$	0·4, 0·3	0·8, 0·7	0·7, 0·6	0·6, 0·5	0·9, 0·8
	Controls and rabbits injected with D.Rn (18) at birth: $t>$	0·231	0·158	1·511	2·914	3·528
	Controls and rabbits injected with D.Rn (18) at birth: $>P>$	0·9, 0·8	0·9, 0·8	0·2, 0·1	0·05, 0·02	0·01, 0·001

(*b*) Comparison within litters.

Inhibiting dose n (μg. H.A./0·15 ml.)		10	20	40	80	500
x_n	Controls (from litters 373, 262)	0·180	0·375	0·585	0·650	0·687
	D.R.S. (from litters 373, 262)	0·172	0·500	0·590	0·633	0·967
x_n (D.R.S.) − x_n (controls) as a function of log n	Δx_n	−0·008	0·125	0·005	−0·017	0·279
	regression of Δx_n on log n	*Not significant* ($F = 2·86$; 1, 3 degrees of freedom)				
x_n	Controls (from litter 194)	0·640	1·250	1·600	1·700	1·700
	D.Rn (18) (from litter 194)	0·252	0·523	1·510	2·277	2·775
x_n (D.Rn (18)) − x_n (control) as a function of log n	Δx_n	−0·388	−0·682	−0·090	0·577	1·075
	regression of Δx_n on log n	*Significant* at the 5 per cent level ($F = 19·05$; 1, 3 degrees of freedom)				

*x_n = log (t_n/t_o), where t_n = titre in the presence of nx μg. of H.A., n = μg. of inhibiting H.A./0·15 ml. of serum, $x = t_o/10·000$, t_o = titre in absence of inhibitor.

The response to D.Rn (18) *of animals injected immediately after birth with D.H.A. and of controls*

In the preceding experiments rabbits had been injected with D.R.S. and D.Rn (18) immediately after birth, and their subsequent response to D.H.A. had been found to differ quantitatively very little from that of controls. We had no direct information on the effect of the injection on the response of the adult animal to the antigen D.R.S. and D.Rn respectively. Thus a piece of evidence important for the evaluation of results was missing. Since information existed on the adult response to D.H.A. of rabbits injected with D.H.A. at birth, the injection of these rabbits with another protein coupled with *p*-diazo-benzene-sulphonic acid was of particular interest.

Ribonuclease which, administered intravenously, had been a fairly good antigen in wild rabbits was a very bad antigen in white Himalayans. It was therefore not surprising that repeated courses of injections with D.Rn (18) given to normal white Himalayans failed to produce an antibody response in six out of seven normal animals.

In spite of these poor prospects, animals from two litters of white Himalayans, some of which had been injected with D.H.A. at birth and whose response to D.H.A. is recorded in Table II, were given courses of injections with D.Rn (18). Most of them formed an antibody which agglutinated tanned cells sensitised with D.Rn (7) (Table VII). After the fourth course of injections with D.Rn (18) had been given to the rabbits of litter 146, two controls and one of the animals injected at birth with D.H.A. had failed to form an antibody against D.Rn. It was thought possible that this relatively high degree of responsiveness was connected with the previous immunisation with D.H.A. This was supported by the unresponsiveness of rabbit 146F, the only rabbit of litter 146 not previously immunised with D.H.A., but contradicted by the unresponsiveness of the control 146G and of 146A which had given, though injected at birth with D.H.A., the highest and most persistent response to D.H.A. when compared with his similarly injected litter mates 146B and C.

TABLE VII.—*The Response to D.Rn* (18) *of Some Rabbits Injected at Birth with D.H.A. and Controls*

All the animals received several courses of injection with D.H.A. during their adult life and before they were injected with D.Rn.

(*a*) Intraperitoneal injections immediately after birth.

Litter	146										263	
Details of injection	(See Table II)											
(*b*) Dose injected into adult rabbits.												
Total mg. D.Rn (18) injected (at intervals of 48 hr.)	3×5	3×4·5	3×5		1×8			3×10 D.H.A.	3×5	2×10	3×5	3×10
(*c*) Agglutination titre of tanned erythrocytes sensitised with D.Rn (7).												
Bleedings†: days after birth	205	225	262	284	286*	289	295	305	374	491	330	366
Rabbits A	**<5**	**<5**	**<5**	**<5**	**<5**	**<5**	**<5**	**<5**	**200**	**200**	§	§
B	**<5**	**200**	**2000**	**5000**	**<5**	**200**	**50**	**20**	**1000**	**5000**	§	§
C	**<5**	**<5**	**<5**	**<5**	**<5**	**200**	**500**	**<5**	**200**	**500**	**50**	**500**
E	§	§	§	§	§	§	§	§	§	§	**<5**	**20**
F	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	§	§
G	<5	<5	<5	<5	<5	<5	<5	<5	50	50	§	§
M	<5	200	500	<5	<5	<5	200	10	1000	1000	<5	<5

* Injection : 284 days after birth.

† Bleedings : 11 days after the last injection of the first course and 5 days after the last injection of each subsequent course.

§ Not available.

Titres in heavy type have been determined on bleedings from rabbits injected with D.H.A. immediately after birth.

A course of injection with D.H.A. was, therefore, given to all the rabbits of litter 146 and the following observations were made :

1. The injection with D.H.A. did not demonstrably affect the titre to D.Rn.

2. A subsequent course of injections with D.Rn produced a response in 146F which was so transient and so weak as to be negligible.

3. 146G and 146A showed a response to D.Rn after a subsequent course of injections with D.Rn.

The last of these observations could not be linked with the preceding injections with D.H.A. since 146A and 146G had received and responded to numerous injections with D.H.A. before the injections with D.Rn (18) had been started. Furthermore the antibody to D.H.A. formed by 146G before the first courses with D.Rn had been given was analysed by agglutination-inhibition and had been demonstrated to contain a high percentage of antibody to D.H.A. which could not be inhibited by H.A. but only by D.H.A.

The first and the second observations, though not excluding the view that the response to D.Rn might be in some way mediated by a preceding response to D.H.A., did not support it. It was finally abandoned when it was found impossible to elicit a response to D.Rn (18) in rabbits from litter 194 when this antigen was injected after a preceding course of injections with D.H.A. Moreover it was found in litter 263 that rabbit 263C, which had been shown to have acquired complete immunological tolerance to D.H.A., responded nevertheless to D.Rn (18).

On balance these experiments can thus be taken to support the previous conclusion that the injection immediately after birth of an azo protein containing 3 per cent azo groups does not inhibit antibody response to another azo antigen which is injected during adult life.

It is difficult to arrive at a reliable qualitative estimate of the antibody specificity of these antisera to D.Rn (18). Agglutination-inhibition has to be carried out with tanned cells sensitised with D.Rn (7). Thus only a fraction of the total antibody can be considered. Inhibition of this agglutination by Rn and D.Rn (18) respectively did not show a difference between the control and the animals injected with D.H.A. immediately after birth (Fig. 6). There was therefore no evidence that injection of D.H.A. during the first days of life had any effect on the response to D.Rn (18) of the adult rabbit.

DISCUSSION

Acquired immunological tolerance has been defined by Billingham, Brent and Medawar (1953, 1955) as a primary failure of the machinery of immunological response. It can be induced by the maintenance of a critical level of foreign proteins during the first days after birth (Hanan and Oyama, 1954 ; Dixon and Maurer, 1955 ; Cinader and Dubert, 1955).

A number of recent publications have reported unsuccessful attempts to induce acquired tolerance to foreign antigens. To evaluate such findings the following factors may have to be considered : (1) The quantity of single antigens injected. (2) The number, route and time of injections. (3) The method of assay of the antibody, and the ability of the assay to distinguish between different antigens that may be injected in a mixture and of which some but not all may have induced tolerance, and possibly (4) the structural similarity of the foreign antigens to homologous macromolecules of the injected animal (cf. Billingham and Brent, 1956).

Our finding that a single injection of 5 mg. of H.A. within the first twelve hours after birth did not lead to the acquisition of immunological tolerance may appear to conflict with the report of Dubert and Paraf (1957) who observed that immunological tolerance was acquired when a much smaller total quantity of H.A. was injected in daily doses during the first eight days of life. Though genetic differences between strains of rabbits might account for these discrepancies,

it is equally probable that to establish acquired immunological tolerance a fairly constant level of antigen has to be maintained within the body fluids during the first week after birth so that repeated injections of antigen might be much more effective than a single injection of the same quantity.

Most of the reported failures to induce tolerance were met with complex mixtures of antigens, foreign erythrocytes or bacteria. The heterogeneity of the antigen may contribute to negative results. In cellular material the usual assay of antibody depends on a small number of the many antigens contained

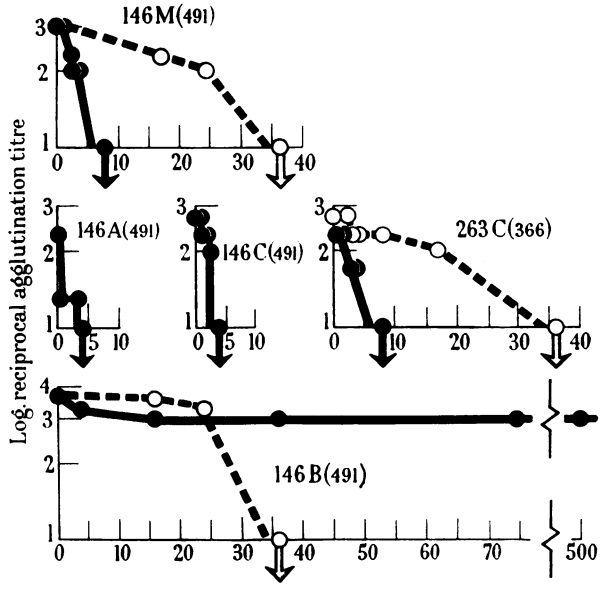

FIG. 6.—The inhibition by Rn and D.Rn (18) of agglutination-erythrocytes sensitised with D.Rn (7).

D.Rn (18) antisera from a normal rabbit (146M) and from rabbits injected at birth with D.H.A. (see Tables II and VII). Values in brackets give the age of the donor in days except for the mother (M) where the number in brackets gives the days after birth of her litter.

●---● agglutination inhibited by Rn.

○——○ agglutination inhibited by D.Rn (18).

Arrows indicate reciprocal titre less than 10.

Abscissa scale indicates μg inhibiting Antigen/0·15 ml. Antiserum.

in the cell. The only antigens reactive in an agglutination test, for instance, are those present on the surface of the cells and representing a minute fraction of the total weight injected. Moreover the weight of cellular material injected into the new-born animals was frequently chosen with reference to the immunising dose of the antigen. The immunising dose may, however, not bear any relation to the dose conferring acquired tolerance. It appears unlikely to do so, if it is considered that whilst immunisation can be effective if only a small number of antibody-forming cells encounter the antigen, acquired tolerance is, for the same reason, best demonstrated if the vast majority of the antibody-forming cells of a new-born animal encounter the antigen. In spite of these reservations, it is

conceivable that the similarity of a foreign protein to the proteins of the recipient affects the ability of the foreign protein to cause acquired immunological tolerance. It is, therefore, of some interest that an azo protein (D.H.A.) may give rise to acquired tolerance.

The acquired immunological tolerance to D.H.A. was not always complete ; the most distinctive feature of the feeble residual response was its transient nature as judged by agglutination tests. Repeated injections into normal animals led to a gradual increase of antibody response, and after several courses of injections to a stabilisation or relative decline of the response. In the partially tolerant animal the peak response usually followed the first course of injections, subsequent courses eliciting less and less agglutinating antibody. Though this observation may seem reminiscent of immune paralysis it would be hazardous to identify it with this phenomenon since elimination studies with H.A. have shown the persistence of a weak immunological response. Thus a change in properties of antibodies rather than inhibition of antibody formation may have occurred during prolonged immunisation.

The small difference in responsiveness to H.A. of animals injected with H.A. at birth and to D.H.A. of animals injected with D.H.A. at birth may be attributable to the removal of D.H.A. by some constitutive process and by the relatively small dose of D.H.A. which reaches the cells mediating the acquisition of tolerance. Should this be the explanation, we would expect that the difference would be apparent and not real and that the injection of small doses of H.A. would produce an effect similar to that described for D.H.A. This feeble response to H.A. could only be demonstrated by elimination studies and not by titration of antibody. The prolonged persistence of H.A. in the circulation would mask the type of response to D.H.A. detected by agglutination tests in the serum of adult animals which had been injected with D.H.A. immediately after birth.

We have so far discussed the response to D.H.A. of adult animals which had been injected immediately after birth with D.H.A. It remains to consider the effect on the later response to D.H.A. of the injection into new-born animals of antigens sharing some chemical determinant with D.H.A. Three different responses were encountered by comparison with D.H.A. antibody from normal controls :

1. In rabbits injected with D.R.S. at birth neither a decrease in the titre nor a change in the specificity of D.H.A. antibody could be demonstrated.

2. In rabbits injected with D.Rn (18) at birth there was no decrease in the titre of D.H.A. antibody, there was, however, a slight but significant effect on specificity (Figs. 3 and 5).

3. In rabbits injected with H.A. at birth the antibody to D.H.A. differed in titre and specificity ; the antibody was mainly adapted to the modified part of the protein (Fig. 2).

The difference between the effect of D.Rn (18) and D.R.S. could be due either to the protein moiety or to the chemical modification.

The similarity between H.A. and D.H.A., the two antigens differing by only 3 per cent of their weight, accounts for the profound effect of the injections of H.A. at birth on the response to D.H.A. of the adult animal.

It may be assumed that

(*a*) Immunological tolerance is acquired to homologous proteins during the normal development of an animal.

(*b*) This naturally-acquired tolerance influences the adult response to heterologous antigens to the extent to which the foreign antigen shares antigenic determinants with any homologous protein, thus limiting the number of effective determinants of most heterologous antigens.

Natural proteins have several determinants, if only a few determinants were shared between homologous and heterologous protein this would be reflected by the specificity of the antibody, if many or most groups were shared the foreign protein would be a " poor " antigen.

The elimination of H.A. from the blood stream of rabbits was determined between the 5th and 11th day after the last of three injections. Rabbits which had acquired immunological tolerance to H.A. or D.H.A. eliminated H.A. with a half life* of 5·12 $\pm$ 0·22 and 5·6 $\pm$ 0·25 days. The values determined by immunochemical methods are in good agreement with values (5·40 $\pm$ 0·25) obtained in tolerant animals in which the elimination of I^{131}-H.A. had been followed by measuring radioactivity and is considerably higher than the value of 3·95 $\pm$ 0·22 days found in normal animals (Cinader and Dubert, 1956). The half life of human albumin (prepared by fractionation with ether) in normal rabbits is confirmed independently by a recent measurement (4·1 $\pm$ 0·4 ; Weigle, 1957 ; HA prepared by fractionation with alcohol).

The difference between the half life of H.A. in normal and in tolerant rabbits may indicate the possible existence of a specific process of elimination which acts before the final rapid elimination of antigen by antibody begins. This process may be abolished by injection of the antigen immediately after birth. Half lives of foreign proteins may have to be determined in tolerant animals if they are to indicate the rate of elimination in the absence of immune processes and if they are to be comparable with half lives of homologous proteins. These conclusions must remain tentative until a satisfactory explanation has been found for variations in half lives determined in different laboratories in similar proteins prepared by different methods (solvent and salt-fractionation).

SUMMARY

A single injection of 5 mg. of human albumin or of 5 mg. of azo human albumin given to rabbits within 12 hr. after birth did not induce acquired immunological tolerance. Acquired immunological tolerance to D.H.A. was induced by four daily injections of 10 mg. D.H.A. or by 20 daily injections of 1 mg. D.H.A. starting within 12 hr. after birth. The elimination of H.A. from the blood stream of animals tolerant to H.A. and D.H.A. was measured by precipitin tests and was found to be 5·1 $\pm$ 0·2 and 5·6 $\pm$ 0·25 days. The mean of the half life of H.A. measured by immunological methods and by radioactive methods in animals with acquired tolerance to H.A. and D.H.A. was 5·35 $\pm$ 0·14, a value considerably higher than that observed in normal animals. A very feeble antibody response to injections with D.H.A. during adult life was observed in some rabbits which had acquired immunological tolerance to D.H.A. This response as measured by agglutination decreased and usually disappeared during subsequent courses of immunisation but could still be demonstrated by the increased rate of elimination of H.A. from the blood stream. An animal tolerant to H.A. was found to respond to D.H.A.

* The half life of rabbit albumin (prepared by fractionation with ethanol) in rabbits has been measured as 5·7 $\pm$ 0·3 by Dixon, Maurer and Deichmiller (1953).

by the formation of antibody primarily adapted to the modified determinants. This brings the total of such responders (Cinader and Dubert, 1956) to three out of eight animals. Rabbits injected at birth with D.R.S. or D.Rn responded during adult life to D.H.A. with an antibody titre similar to that of animals not injected at birth.

Rabbits injected with D.Rn at birth produced antibodies to D.H.A. which were slightly less well adapted (significant at the 5 per cent level) to the modified determinants of the antigen than antibodies produced by the controls.

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