THE NATURE OF ANTIGEN-ANTIBODY COMPLEXES FORMED IN RABBITS DURING AN IMMUNE RESPONSE TO BOVINE SERUM ALBUMIN*, ‡

By WILLIAM O. WEIGLE, PH.D.§

(From the Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh)

(Received for publication, January 15, 1958)

The dynamics of the interaction of serum protein antigens and antibody during a rabbit's immune response have been studied in considerable detail, using I^{131}-labelled antigens (1, 2). These studies indicate that as antibody is formed by the host, it combines with circulating antigen and these antigen-antibody complexes are removed from the circulation, more or less, rapidly. Following this elimination of the complexes, free antibody appears in the circulation and I^{131}-labelled antigen is no longer detectable in the blood. However, a series of publications has reported that complexes of such serum protein antigens (3, 4) or antigen-like materials (5, 6) and the homologous antibody may persist in the circulation of the rabbit long after the appearance of free antibody. The present studies were undertaken to help resolve this difference, and also to obtain precise knowledge of the development of serum protein antigen-antibody complexes and their fate *in vivo*. Knowledge concerning the *in vivo* formation and fate of these complexes is particularly important since they have been implicated in the immunopathologic changes associated with the injection of such antigens (7–9).

Materials and Methods

I^{131}-Labelled Antigen.—Bovine serum albumin (BSA), Armour and Co., Lot P67908, was labelled with I^{131} (I*) by the method described by Talmage *et al.* (1).

X-Radiation.—Some of the rabbits were given 400 r whole body x-radiation 48 hours prior to the injection of antigen. X-radiation was done at 200 kvp and 15 ma. with a filter of 1 mm. Al and 0.25 mm. Cu delivering at a rate of 16 r per minute at the target distance.

Injections and Bleedings.—Fifteen normal, and 7 x-radiated albino rabbits weighing approximately 2.5 kg. were injected intravenously with 100 mg. of I*BSA and then bled (8 to 15 ml. of blood), periodically. Seven of the normal rabbits were later given another intravenous injection of I*BSA (50 mg.) and again bled periodically.

* Supported by the United States Atomic Energy Commission Contract No. AT (30-1)-1205.

‡ Publication 145 of the Department of Pathology, University of Pittsburgh School of Medicine.

§ Public Health Service Research Fellow of the National Institute of Neurological Diseases and Blindness.

Analyses of Sera.—The sera were separated from the blood and analyzed for I*BSA, anti-BSA, alkali-precipitable material (3), and I*BSA bound to globulin (antibody).

The amount of I*BSA remaining in the blood of rabbits was determined by counting the protein-bound (precipitated with 10 per cent trichloroacetic acid) I* activity of the sera in well type gamma counters. Sufficient counts were obtained to insure a maximum counting error of no greater than 5 per cent.

Anti-BSA was determined by the quantitative immunochemical technique developed by Heidelberger and coworkers (10). Before the sera were analyzed for antibody they were decomplemented with 100 mg. N per ml. of an unrelated antigen–antibody precipitate (11).

It was possible to detect the presence of circulating I*BSA which was bound to globulin by employing the ammonium sulfate precipitation technique of Farr (12). The sera containing I*BSA were fractionated at 50 per cent ammonium sulfate saturation at 0°C. This treatment precipitates the serum globulin plus any I*BSA which is combined with it. I*BSA not combined with the globulin remains in the supernatant. It is assumed that the I*BSA precipitated at 50 per cent ammonium sulfate saturation is bound to antibody globulin and present in the sera in the form of soluble I*BSA–anti-BSA complexes.

Sera were tested for alkali-precipitable material by the method described by Sternberger (3). Three ml. of the sera were diluted with an equal volume of 0.15 M NaCl and adjusted to pH 12.6 at 1°C. with NaOH and allowed to remain at 1°C. for 6 minutes, and then readjusted with HCl to the original pH. The readjusted sera were made up to 4 volumes with 0.15 M NaCl and 1:10,000 merthiolate, and incubated for 2 hours at 37°C., and then stored for 4 to 10 days at 0–3°C. The precipitates which formed were washed twice with 8 ml. of 0.15 M NaCl, dissolved in NaOH, and then made up to known volumes in volumetric flasks. Aliquots were then removed with volumetric pipettes for N analyses. The I^{131} content of alkali-precipitable material, obtained from sera which also contained I*BSA, was determined before the precipitates were dissolved with NaOH. The ability of excess BSA to dissolve alkali-precipitable material was tested. Precipitates obtained from 1 to 3 ml. of several of the sera were finely suspended and transferred to 5 ml. volumetric flasks. The suspensions were made up to volume and 1 ml. aliquots transferred, with volumetric pipettes, to 4 ml. conical centrifuge tubes. 2.4 mg. of BSA N were added to 2 of the tubes and an equal volume of 0.15 M NaCl was added to the other 2 tubes. The 0.15 M NaCl and the BSA solution were buffered at pH 7.2, with phosphate buffer, and contained 1:10,000 merthiolate. After incubation at 37°C. for 1 hour, and then storage at 0 to 3°C. for 2 days, the precipitates were washed twice with 2 ml. of 0.15 M NaCl and the total N was determined. The Markham modification of the micro Kjeldahl technique was used for all N determinations (13).

RESULTS

*Serological Changes Following Intravenous Injections of I*BSA into Rabbits.*—I*BSA (100 mg.) injected into normal rabbits, after equilibration between intra- and extravascular fluids, was eliminated at a slow logarithmic rate until the 7th to 9th day, when a rapid immune elimination of the I*BSA began in most of the animals (Table I). The maximum concentration of antibody appeared in 2 to 4 days after the disappearance of I*BSA from the blood. Similar results have been previously reported by Talmage *et al.* (1). In most of the animals a portion of the circulating I*BSA was bound to serum globulin at the same time that immune elimination of the antigen began. The per cent of the I*BSA bound to globulin increased until the antigen was almost completely eliminated from the blood. Several animals showed decreases in the per cent of the I*

TABLE I

*Serological Changes Following Intravenous Injections of I*BSA into Rabbits*

Days following first injection of I*BSA (100 mg.).

Rabbit No.		1	3	5	7	8	9	10	11	12	14	16	Maximum antibody N* mg.	17‡	19	21	23	25	28	Maximum antibody N* mg.
46-70	A	35.0	21.4	14.4	11.8		4.4	0.3	0.1	0			98.4	26.8	19.5	0.3	0			
	B	1.9	1.2	0.9	2.3		27.4	77.3						32.6	10.4	22.5				198.9
	C	0	0	0	0		0	0	0	0	38.9	69.0		0	0	0	0	0	0	
46-71	A	44.2	23.1	17.4	13.9		10.9	7.3	5.3	3.6	0.28	0	43.6	29.8	22.3	0.8	0			
	B	3.2	2.9	1.1	2.8		16.5	47.5	51.9	96.2	86.7			51.0	40.1	27.7				105.1
	C	0	0	0	0		15.5	219		429	658	528		530	296	670	843	555	0	
46-72	A	40.1	22.8	17.1	10.3		3.8	1.0	0.1	0			33.3	29.0	29.0	19.0	0	0		
	B	2.1	1.7	1.5	4.1		36.8	75.1	62.5					23.2	11.8	46.0				57.2
	C	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0	
46-73	A	30.2	24.0	17.3	10.6		4.7	1.5	0.1	0			74.8	28.3	9.5	0	0			
	B	2.2	2.7	0.9	2.9		16.5	61.8	47.0					41.6	15.8					121.6
	C	0	0	0	0		0	0	0	0	0	0		0	0	0	701	268	0	
46-74	A	39.5	22.0	13.9	7.5		0.2	0					114.8	25.4	8.3	0	0			
	B	2.7	3.6	1.4	6.7		40.7							45.7	18.5					200.4
	C	0	0	0	0		0	0	0	0	0	0		0	0	0	869	861	0	
46-75	A	40.4	23.9	15.6	9.9		3.8	0.1	0				84.0	27.3	16.4	0.2	0			
	B	3.7	2.0	1.4	6.3		60.0	41.7						59.5	38.0	40.2				129.7
	C	0	0	0	0		0	0	0	0	0	0		0	0	616	883	840	0	
46-76	A	39.4	20.9	11.5	5.0		0.3	0					178.4	29.7	15.5	0.1	0			
	B	2.1	1.0	1.9	9.8		35.1							28.4	10.3					249.8
	C	0	0	0	0		0	0	0	0	0	0		0	0	0	0	0	0	
47-67	A		27.1	15.9		4.9		0					97.2							
	B		1.7	2.2		48.7														
	C		0	0		0		0	0	0	0	0								
47-68	A		24.1	19.5		6.6		0					79.1							
	B		1.6	1.2		30.4														
	C		0	0		0		0	0	0	0	0								
47-69	A		28.8	20.8		5.6		0.1	0	0	0		60.4							
	B		1.3	2.4		45.0														
	C		0	0		0		0	0	26.6	0	0								
47-70	A		29.3	16.8		8.4		5.3	1.2	0.1	0		20.2							
	B		1.2	1.8		12.4		22.2	92.5											
	C		0	0		0		0	0	0	0	0								
47-71	A		27.0	22.9		9.2		3.9	2.5	0.1	0		17.1							
	B		1.2	1.4		18.5		40.2	68.2											
	C		0	0		0		0	0	0	0	0								
47-72	A		18.6	12.7		5.2		0.1	0				84.6							
	B		1.6	2.4		31.0		52.0												
	C		0	0		0		0	0	0	0	0								
47-73	A		30.0	20.6		10.3		4.5	2.1	0.2	0		12.3							
	B		3.2	2.6		15.8		40.1	68.7											
	C		0	0		168		30.4	10.1	0	92.0	0								
47-74	A		16.9	11.7		5.2		0.2	0				48.2							
	B		3.4	2.7		35.6		91.0												
	C		0	0		244		39.4	0	0	0	71.0								

*Maximum concentration of antibody was found in the sera 2 to 4 days after complete antigen elimination.

‡ Seven of the rabbits received a second injection of I*BSA (50 mg.) on the 17th day following the first injection. The blood analyzed on the 17th day was collected 2 hours following the injection of I*BSA.

A, per cent I*BSA injected remaining in total blood volume.

B, per cent I*BSA remaining in total blood volume precipitable with 50 per cent $(NH_4)_2SO_4$ saturation.

C, mg. N precipitated/ml. serum after treatment with alkali (3).

bound to globulin when the circulating protein-bound I* fell below 0.3 per cent. This might have been the result of the persistence of trace amounts of contaminating I* serum proteins. The I*BSA preparation contained approximately 1 per cent of bovine proteins other than albumin and these trace contaminants may have remained in the circulation after the immune elimination of the albumin.

Two hours after a second injection of I*BSA (50 mg.), 20 to 60 per cent of the circulating antigen was bound to serum globulin, presumably antibody persisting from the previous response. The fall in protein-bound I*BSA between 2 hours and 2 days and the subsequent rise between the 2nd and 4th day was probably the result of elimination of the complexes formed with persisting antibody and then an increase in complexes arising from the reaction of newly synthesized anti-BSA with the remaining I*BSA.

Five of the 15 normal rabbits receiving a primary injection of I*BSA developed alkali-precipitable material in their sera, which first appeared between the 8th and 14th day following the injection. When 7 of these rabbits (2 of which had alkali-precipitable material after the first injection) (Table I) were given a second injection of I*BSA, 17 days after the first, 3 additional animals developed alkali-precipitable material in their sera. The alkali-precipitable material appeared in the sera of these 3 rabbits 4 to 6 days following the second injection of antigen. One rabbit (No. 46-70) whose serum contained alkali-precipitable material after the first injection did not have this material in its serum after the second injection. There was no correlation between the presence or absence of alkali-precipitable material and the maximum antibody concentration. Seven rabbits receiving 400 r whole body-radiation 2 days prior to the injection of 100 mg. of I*BSA and not showing an immune elimination of the antigen, contained neither circulating I*BSA–anti-BSA complexes nor alkali-precipitable material in their sera.

Fig. 1 is a graphic representation of serological changes following injections of I*BSA into a rabbit. Immune elimination of the initial injection of antigen began shortly after the 7th day and was completed by the 11th day. It can be seen that with the appearance of antigen-antibody complexes in the serum, immune elimination began and that the per cent of circulating I*BSA bound to the globulin increased with time. Free anti-BSA was first observed in the serum shortly after the elimination of circulating I*BSA. A second injection of I*BSA on the 17th day was followed by a rapid drop in circulating antigen as a result of the simultaneous combination of I*BSA with persisting antibody and equilibration of I*BSA between intra- and extravascular fluid spaces. Rapid immune elimination of the remaining I*BSA, beginning between the 2nd and 4th day, was the result of newly synthesized antibody. Alkali-precipitable material was present in the serum of this animal on the 14th and 16th day following the first injection but not after the second injection. The pattern of both antigen elimina-

tion and appearance of circulating antigen-antibody complexes seen in this animal is representative of most of the animals studied. However, a rabbit (No. 46-71) which eliminated the antigen more slowly, gave somewhat different results (Fig. 2). This rabbit did not eliminate the first injection of I*BSA until the 15th or 16th day, and circulating antigen-antibody complexes were detected over a 5 day period. The complexes appeared in the serum by the 9th day, before the immune elimination of I*BSA was initiated, and on the 12th day almost all of the circulating I*BSA was bound to globulin. Alkali-precipitable material

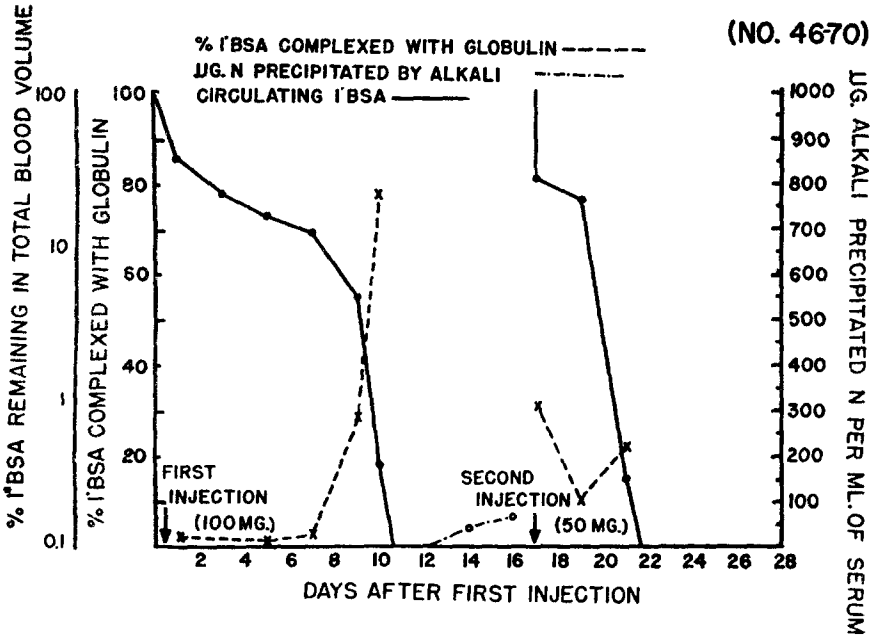

FIG. 1. Serologic changes following injections of I*BSA into a rabbit

was detected in large amounts during the immune elimination of antigen. This material was also detected in the serum after the second injection of antigen, reaching a peak on the 6th day and disappearing by the 11th day after the injection.

One would not necessarily expect the complexes, precipitated with ammonium sulfate at 0°C., to be representative of the complexes as they circulate in the blood at 37°C. In order to obtain a more authentic picture of the complexes as they do exist *in vivo*, the complexes in some of the sera were also precipitated by ammonium sulfate at 37°C. (Fig. 3). Somewhat less of the I*BSA was combined with globulin at 37°C. than at 0°C. This was probably the result of a greater dissociation of complexes at the higher temperature.

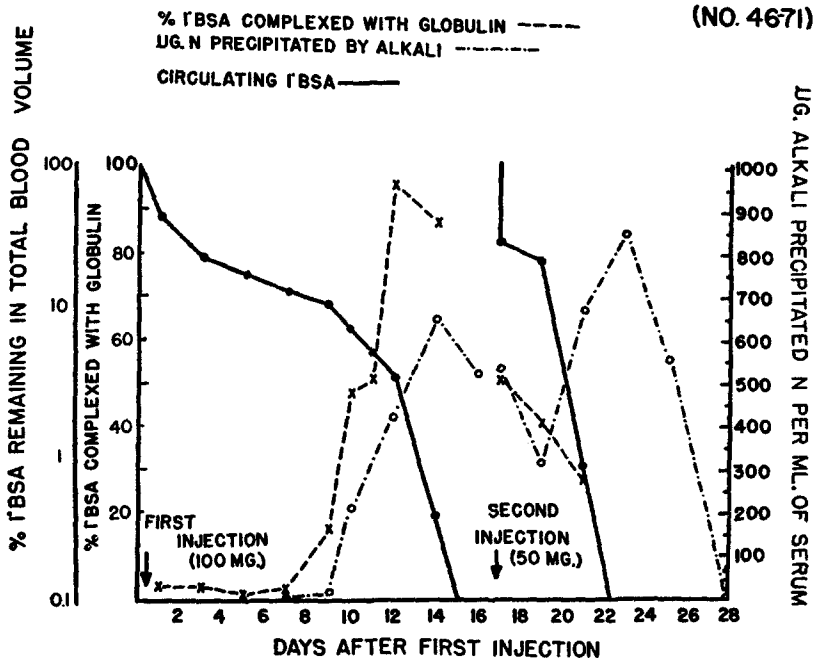

FIG. 2. Serologic changes following injections of I*BSA into a rabbit

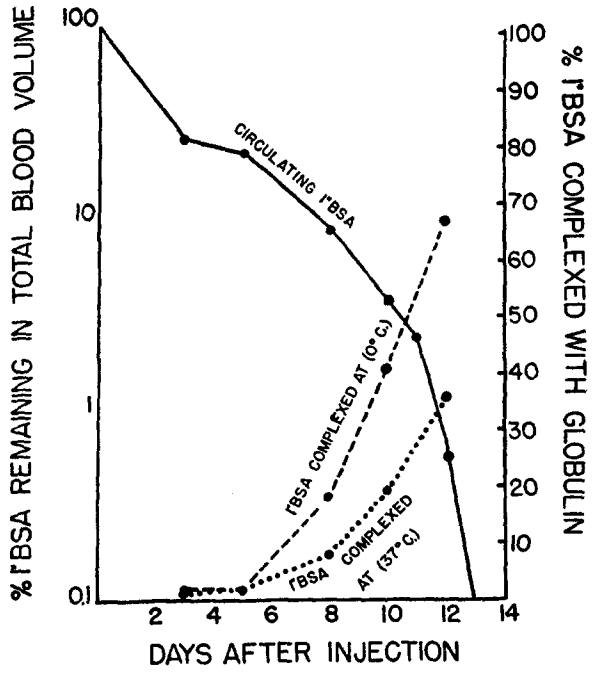

FIG. 3. I*BSA complexed with serum globulin

*Circulating I*BSA Precipitated by Alkali and Ammonium Sulfate.*—Four of the 15 normal animals, injected for the first time with I*BSA, contained both alkali-precipitable material and I*BSA in their sera at the same time. In order

TABLE II

*Circulating I*BSA Precipitated by Alkali and Ammonium Sulfate*

Rabbit No.	Day following 1st injection of I*BSA‡	Protein pptd. by alkali treatment		Percentage circulating I*BSA pptd. by	
		Total N/ml. sera μg.	BSA N/ml. sera μg.	Alkali treatment	Ammonium sulfate
46-71	9	15.5	0	0	16.5
46-71	10	219	0.48	0.7	47.5
46-71	12	429	0.27	2.1	96.2
46-71	14	658	0.09	2.9	86.7
46-71	17§	530	3.15	2.3	51.0
46-71	19	296	1.56	1.9	40.1
47-73	8	168	0.62	0.6	15.8
47-73	10	30.4	0.11	0.2	40.1
47-74	8	244	0.50	0.8	35.6
47-74	10	39.4	0.02	0.4	91.0
46-75	21	616	0.05	1.8	40.2

‡ The second injection of I*BSA was given on the 17th day.

§ This bleeding was made 2 hours after the second injection of I*BSA.

TABLE III

Effect of Antigen on the Solubility of Alkali-Precipitable Material

Rabbit No.	Day following first injection of I*BSA	Total N remaining in alklali ppts.	
		After incubation* with 0.15 M NaCl	After incubation* with 2.8 mg. BSA N
		μg.	μg.
47-71	12	172	164
47-71	23‡	162	176
46-75	25	159	142
47-73	8	126	113
47-74	8	118	129

* Precipitates were incubated at 37°C. for 1 hour and then at 0–3°C. for 48 hours.

‡ The second injection of I*BSA was given on the 17th day.

to determine whether the alkali-precipitable material contained I*BSA, the precipitates resulting from alkali treatment were washed with 0.15 M NaCl and their I* activity determined. The amount of I*BSA as determined from I* radioactivity is shown in Table II. Only a very small per cent of the circulating

I*BSA was present in the alkali-precipitable material even when a large per cent of it was bound to serum globulin as determined by ammonium sulfate precipitation. The μg. of I*BSA N which was precipitable by alkali treatment was extremely small in comparison to the total μg. of N precipitated. If one assumes for the moment that the alkali-precipitable material was antigen-antibody complexes as has been reported, and calculates antibody N/antigen N ratios from the above values, one arrives at ratios between 150 and 10,000, which are in complete disagreement with the known properties of I*BSA–anti-BSA precipitates.

Dissolution of Alkali-Precipitable Material with Excess Antigen.—The alkali-precipitable material from five sera of 4 different rabbits was incubated both in the presence and in the absence of BSA. The amount of N remaining in the precipitates was approximately the same in both instances (Table III). Under the conditions of the experiment and with the concentration of BSA employed, it appeared that excess antigen was not able to dissolve the alkali-precipitable material.

DISCUSSION

It has been postulated that once antibody to a serum protein–antigen is synthesized it combines with the antigen in the blood, and the resulting complex is then rapidly removed from the circulation (1). In the present experiments a rapid immune elimination of the antigen began, in most of the rabbits, at about the same time that antigen-antibody complexes appeared in the sera. The immune elimination of antigen from the blood of most rabbits, presumably in the form of complexes, takes only a few days. However, complexes were present in the sera of one rabbit (No. 46-71), 1 to 2 days prior to the rapid immune elimination of the antigen. It seems likely that once antibody is synthesized it attaches to antigen, forming antigen-antibody complexes in the body fluids.

Early in antibody formation these complexes are formed in conditions of great antigen excess and as has been reported elsewhere, such complexes can circulate for some time in the blood (14). As more antibody is synthesized, the antibody/antigen ratio of the complexes increases, thus increasing the size of the complexes until they are rapidly removed from the circulation. If this is the case, then the rate of antibody synthesis, the amount of antigen injected, and perhaps the quality of antibody produced would determine the rate of immune elimination of antigen. Complement may also affect the immune elimination of antigen since it is capable of both reacting with and precipitating soluble protein-rabbit antiprotein complexes *in vitro* (15). The sequence of complex formation and antigen elimination in rabbits injected with 100 mg. of I*BSA is similar to that seen in guinea pigs injected with 3 to 4 mg. of I* rabbit serum albumin, although both events take place somewhat earlier in rabbits (16).

There is little relationship between the alkali-precipitable material described

by Sternberger (6) and the I* antigen-antibody complexes precipitated by ammonium sulfate. The ability of excess specific antigen to dissolve the alkali-precipitable material was used as evidence that this material consisted of antigen and specific antibody (5). In the present study, it was not possible to dissolve the alkali-precipitable material from 4 different sera with excess antigen. The amount of excess antigen used was at least 10 times that necessary to completely dissolve the same amount of immune precipitates which were prepared from BSA and rabbit anti-BSA. However, Sternberger *et al.* reported dissolution of alkali-precipitable material when much larger amounts of excess antigen were employed (4). At times, when both alkali-precipitable material and I*BSA were present in sera, significant quantities of the I*BSA were never found in the alkali-precipitable material, although a large per cent of the I*BSA was bound to serum globulin.

Sternberger and Dixon have observed a great discrepancy between the amounts of I*-labelled bovine gamma globulin (I*BGG) and alkali-precipitable material in the circulation of immunized rabbits (17). Between 4 and 21 days after the immune elimination of I*BGG, I^{131} equivalent to 0.04 μg. of I*BGG persisted per ml. of sera. In these same sera an average of 150 μg. of alkali-precipitable N per ml. of sera was detected. If this alkali-precipitable material had been antibody-antigen complexes, the antibody N/antigen N ratio would have been 3750 or more, which is obviously impossible. Whether this material contains a host–synthesized antigen-like substance, rather than the original antigen, as has been recently suggested (6), remains to be seen. Thus, the *in vivo* work with I*-labelled antigen would seem to eliminate the possibility that alkali-precipitable material is an antigen-antibody complex.

More recently it was proposed that in the alkali-precipitable material, the antigen-like material was bound, in the host, to non-precipitating antibody rather than to precipitating antibody and that these complexes were capable of circulating in the host (6). However, when protein-(non-precipitating)anti-protein complexes were prepared at equivalence, *in vitro*, and injected intravenously into rabbits, the complexes were immediately removed from the sera and did not return (14). On the other hand, these complexes, prepared in a large excess of antigen, circulated in the blood with a half-life of approximately 2 to 3 days, just as do complexes prepared with precipitating antibody and a large amount of excess antigen. It then appears that antigen-(non-precipitating)antibody complexes formed *in vitro* are handled *in vivo* in the same manner as antigen-(precipitating)antibody complexes.

It was further suggested that the reaction of antigen-like material with non-precipitating antibody protects the material against elimination from the circulation and destruction by the host (6). This protection was postulated as being the factor which permits the persistence of antigenic material, and, thus, the continued formation of antibody. However, when protein–anti-protein com-

plexes (containing either precipitating or non-precipitating antibody) were injected into rabbits, these complexes were eliminated from the circulation and catabolized more rapidly than either the antibody or antigen when injected alone (14). Also, native foreign serum proteins, which are capable of stimulating antibody production in the rabbit, have never been demonstrated to persist in that species for long periods of time. A number of workers have demonstrated that chemically modified proteins will persist in the rabbit, but these proteins have been shown to behave much differently in rabbits than the corresponding native proteins (18). Furthermore, Dixon *et al.* (19) and more recently McMaster and Edwards (20) have shown that if there is any persistence of native protein antigens or their derivatives in the rabbit, they are not capable of eliciting significant antibody production in that species.

SUMMARY

The immune elimination of soluble BSA, following an intravenous injection, is accompanied by the appearance of circulating antigen-antibody complexes. The pattern of the appearance of circulating antigen-antibody complexes and the immune elimination of antigen probably depends on the amount of antigen injected, the rate of antibody synthesis, and perhaps, the quality of antibody produced.

There is no relationship between the I* antigen-antibody complexes detected during the immune response in rabbits by ammonium sulfate precipitation and the material precipitated from immune sera as a result of treatment with alkali. Alkali-precipitable material present in the serum of rabbits at a time when I* antigen is also present contain at most only traces of the antigen.

The author wishes to acknowledge the excellent technical assistance of Miss Dorothea Williamson and Mrs. Mary Bloom.

BIBLIOGRAPHY

1. Talmage, D. W., Dixon, F. J., Bukantz, S. C., and Dammin, G. J., *J. Immunol.*, 1951, **67,** 243.
2. Dixon, F. J., *J. Allergy*, 1954, **25,** 487.
3. Sternberger, L. A., Maltaner, F., and DeWeerdt, J., *J. Exp. Med.*, 1953, **98,** 451.
4. Sternberger, L. A., Feinberg, S. M., and Clarke, M. E., *J. Exp. Med.*, 1956, **103,** 523.
5. Sternberger, L. A., *J. Exp. Med.*, 1956, **103,** 545.
6. Sternberger, L. A., *J. Allergy*, 1957, **28,** 40.
7. Germuth, F. G., and McKinnon, G. E., *Fed. Proc.*, 1957, **16,** 413.
8. Germuth, F. G., and McKinnon, G. E., *Bull. Johns Hopkins Hosp.*, 1957, **101,** 13.
9. Dixon, F. J., Vazquez, J. J., Weigle, W. O., and Cochrane, C. G., *Arch. Path.*, 1958, **65,** 18.
10. Kabat, E. A., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1948.

11. Maurer, P. H., and Talmage, D. W., *J. Immunol.*, 1953, **70,** 135.
12. Farr, R. S., *Fed. Proc.*, 1956, **15,** 586.
13. Markham, R., *Biochem. J.*, 1942, **36,** 790.
14. Weigle, W. O., data in preparation.
15. Weigle, W. O., and Maurer, P. H., *J. Immunol.*, 1957, **79,** 221.
16. Weigle, W. O., and Dixon, F. J., *J. Immunol.*, 1957, **79,** 24.
17. Sternberger, L. A., and Dixon, F. J., unpublished data.
18. Dixon, F. J., Bukantz, S. C., Dammin, G. J., and Talmage, D. W., *Fed. Proc.*, 1951, **10,** 553.
19. Dixon, F. J., Maurer, P. H., Weigle, W. O., and Deichmiller, M. P., *J. Exp. Med.*, 1956, **103,** 425.
20. McMaster, P. D., and Edwards, J. L., *J. Exp. Med.*, 1957, **106,** 219.