IMPAIRMENT OF THE ANTIGENICITY OF A PROTEIN ANTIGEN FOLLOWING ITS INJECTION INTO RABBITS

By PHILIP D. McMASTER, M.D., AND JOSHUA L. EDWARDS, M.D.

(From The Rockefeller Institute for Medical Research)

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For several years the fate of protein antigens injected into mice or rabbits has been a matter of interest in this laboratory (1–6). Experiments have shown (4) that liver tissue—taken from "donor" rabbits previously injected with bovine γ-globulin as an antigen and transferred to normal mice—rendered the recipients anaphylactically sensitive to subsequent challenge with the same antigen. This response was obtained with liver taken from the donor rabbits as long as 6 weeks after they had been injected with the protein. Evidently the livers of the donors had retained enough antigen, or antigenic material sufficiently like it, to render the recipient mice anaphylactically sensitive to bovine γ-globulin.

The recipient mice, on the other hand, showed no precipitating antibody in their blood. Nevertheless, the intensity of the anaphylactic reactions of some of them suggested that there might have been enough antigenic material transferred to them in the liver tissue to engender serologically demonstrable amounts of antibody were it not for the fact that mice produce circulating antibody poorly (1–4). What then can be said about the nature of the antigenic material persisting in the livers of the donor rabbits? Was it some sort of degraded material only capable of inducing anaphylactic sensitivity and not able to elicit the formation of complete antibody serologically detectable by the precipitin test? Or was it unchanged antigen present in the transferred liver tissue in amounts too small to elicit detectable antibody formation in the recipients?

Since mice, as just mentioned, form circulating antibody poorly, these questions could not be answered by using these animals as recipients. Therefore, it seemed worthwhile to repeat experiments of this sort using a species of animal that forms antibodies well, namely the rabbit.

General Plan of the Experiments

It was planned, as in the preceding work, to take from donor rabbits previously injected with the antigen, bovine γ-globulin, large amounts of liver tissues presumably containing antigen, whether degraded or not, and to inject repeatedly this material into the peritoneal cavities of other, recipient rabbits. These were to be prepared

by a previous injection of the same antigen in small amounts and left until their blood no longer showed the presence of antibody. Only then were the transfers of liver tissue from the donors to be made, so that the recipient animals would be given a chance to exhibit a secondary reaction. The appearance of specific antibody to bovine γ-globulin in the blood of these animals would indicate that the antigen had persisted in the livers of the donor rabbits in a relatively intact form. By removing the liver tissue from the donors at various intervals of time after they had been injected with antigen, one might be able to learn how long the antigen remained intact in the tissue.

Experimental Procedures

Normal rabbits about 3 kilos in weight, which were to serve as donors, were injected intravenously with 100 mg. of bovine γ-globulin per kilo, in the form of a 5 per cent aqueous solution. Some of these rabbits were bled on the 8th day after injecting the antigen, and at daily intervals thereafter. The disappearance of antigen from the blood and the appearance of antibody in it were determined by precipitin tests, to be described below. Six of these animals were exsanguinated on the 14th day after they received antigen, after 2 or 3 bleedings had shown none of it remaining in their blood and only a little antibody. The livers were removed and treated as will shortly be described. Four other donor rabbits, bled 14 and 17 days after receiving antigen, were sacrificed for liver tissue on the 21st day. These animals showed circulating antibody but no antigen. Two more donors showing no antigen in their sera were similarly treated after 28 days.

In all instances the livers were removed with precautions for asepsis, and washed with chilled 0.85 per cent NaCl solution in a chilled container. Some were immediately homogenized with the addition of saline solution in a chilled, sterile Waring blendor, in a cold room at 5°C. These precautions were taken to inhibit enzymatic changes which might affect any antigen in the tissues. The chilled homogenates were sealed in small tubes, each containing 20 gm. of tissue with added saline, and immediately frozen and stored at −23°C. In other instances the whole livers were immediately stored at the same temperature.

The Transfer of Liver Tissue to Prepared Recipient Rabbits.—From time to time normal rabbits, about 2 kilos in weight, were prepared to act as recipients of the liver tissue. Each of these animals received 5 mg. of bovine γ-globulin intravenously, and they were bled at various intervals. When circulating antibody had disappeared from the sera, pairs of these animals, which were to receive liver, were given, for a week, drinking water containing 50 mg. of terramycin per liter which they drank readily. With strict aseptic precautions 20 gm. of liver tissue, kept at −23°C., as just described, and derived from only 1 donor, was thawed, transferred at once to a chilled container in the cold room and thoroughly ground in a chilled TenBroeck grinder, after the addition of enough saline to bring the homogenate to a total volume of 35 to 40 ml. One hundred units of penicillin, dissolved in saline was added to it and mixed. The recipient rabbit was then injected intraperitoneally while the suspension of tissue was still quite cold, since it had been exposed to room temperature only while being mixed in the injecting syringe. In spite of this, the animals tolerated the injections well. Each recipient received 3 transfers of the kind.

The Detection of Circulating Antigen and Antibody. A Semiquantitative Precipitin Technique.—The presence of antigen and antibody in the sera of both donor and recipient animals was sought by the usual qualitative precipitin techniques, using the customary ring test in tubes 8 mm. in diameter and also the capillary precipitin method. To determine whether antibody was increasing or decreasing in the blood of these animals, a semiquantitative technique was devised which was sufficiently accurate for the purpose. The ordi-

TABLE I

Specimen Protocol of the Semiquantitative Technique

The reactions of double dilutions of 3 antibody-containing fluids, A, B, and C, described in the table, when layered over 6 different solutions of the appropriate antigen.

Fluid A. Whole lyophilized anti-bovine γ-globulin rabbit serum in 0.9 per cent NaCl solution. (464 μg. of antibody protein per ml.)

Tube No.	1	2	3	4	5	6	7	8	9	10	11
Double dilutions of fluid A	Undiluted	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Antibody protein, *μg. per ml*	464	232	116	58	29	14.5	7.2	3.6	1.8	0.9	0.45
When layered over the following solutions of antigen:—											
10 per cent bovine γ-globulin in 0.9 per cent NaCl solution	+	+	+	+	+	0	0	0	0	0	0
1 " "	+	+	+	+	+	0	0	0	0	0	0
0.1 " "	+	+	+	+	+	0	0	0	0	0	0
0.01 " "	+	+	+	+	0	0	0	0	0	0	0
0.001 " "	0	0	0	0	0	0	0	0	0	0	0
0.0001 " "	0	0	0	0	0	0	0	0	0	0	0

Fluid B. Precipitated γ-globulin from anti-bovine γ-gluobulin rabbit serum. (1760 μg. of antibody protein per ml.)

Tube No.	1	2	3	4	5	6	7	8	9	10	11
Double dilutions of fluid B	Undiluted	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Antibody protein, *μg. per ml*	1760	880	440	220	110	55	27.5	13.7	6.8	3.4	1.7
When layered over the following solutions of antigen:—											
10 per cent bovine γ-globulin in 0.9 per cent NaCl solution	+	+	+	+	+	+	0	0	0	0	0
1 " "	+	+	+	+	+	+	+	0	0	0	0
0.1 " "	+	+	+	+	+	+	+	0	0	0	0
0.01 " "	+	+	+	+	+	+	+	0	0	0	0
0.001 " "	+	+	+	+	+	+	+	0	0	0	0
0.0001 " "	+	+	+	+	+	+	+	0	0	0	0

Fluid C. Precipitated γ-globulin from anti-bovine γ-globulin rabbit serum. (480 μg. antibody protein per ml.)

Tube No.	1	2	3	4	5	6	7	8	9	10	11
Double dilutions of fluid C	Undiluted	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
Antibody protein, *μg. per ml*	480	240	120	60	30	15	7.5	3.7	1.8	0.9	0.45
When layered over the following solutions of antigen:—											
10 per cent bovine γ-globulin in 0.9 per cent NaCl solution	+	+	+	+	+	0	0	0	0	0	0
1 " "	+	+	+	+	+	0	0	0	0	0	0
0.1 " "	+	+	+	+	+	0	0	0	0	0	0
0.01 " "	+	+	+	0	0	0	0	0	0	0	0
0.001 " "	+	+	0	0	0	0	0	0	0	0	0
0.0001 " "	0	0	0	0	0	0	0	0	0	0	0

nary serum dilution type of precipitin reaction was modified in such a way that one could attain this end with certainty, as will now be described, using only a single, or at the most two, concentrations of antigen against various antiserum dilutions. For these purposes it seemed best to use a capillary interfacial test in order to avoid the zone phenomena which occur when solutions of antibody and antigen are mixed. Since, by diffusion from the interface optimal proportions for precipitation should present themselves, it would not be necessary, first, to make trials to determine the proper concentration of antigen or antibody solutions to use.

Accordingly, certain antigen and antibody containing fluids, to be described below, were taken up in the usual manner into capillary tubes 10 cm. long and 1.5 mm. internal diameter, to form a column about 6 to 7 cm. in length. Since these tubes were larger in bore than those generally used for capillary tests the interface formed was correspondingly broader than usual, and the larger amounts of the fluids in the tubes formed more precipitate for observation. Readings were made, at the usual intervals up to 96 hours, under a binocular microscope with the tubes held against a dark background while the beam of a strong microscope illuminator played upon them at an angle of 60°. Under these conditions the precipitates in the tubes, magnified about 25 times, stood out like particulate matter in a dark field microscope. All solutions used were cleared either by centrifugation or filtration or both, and appropriate control tubes were, of course, set up.

Three solutions of known antibody protein content, as determined by the method of Heidelberger and Kendall (7, 8) were used to develop the technique. One of these solutions, Table I, fluid A, which contained 464 μg. of antibody protein (74.2 μg. of antibody N) per ml., was made up in 0.9 per cent NaCl solution from lyophylized, whole rabbit antiserum. The other 2, Table I, fluids B and C, containing respectively 1760 and 480 μg. of antibody protein per ml. (181.6 μg. and 76.8 μg. of antibody N) were made up from precipitated antibody globulin obtained as outlined by Kabat and Mayer (8). Twofold dilutions of the three fluids, in 0.9 per cent sodium chloride solution, contained the amounts of antibody protein per ml., shown in the third horizontal row in each part of the table. Capillary precipitin tests were then set up with each of the dilutions against six aqueous solutions of bovine γ-globulin (BGG), ranging in 10-fold dilutions of the protein, from 10.0 per cent to 0.0001 per cent. As the table shows, either 1.0 per cent or 0.1 per cent BGG solutions yielded approximately the same end points when titered against the solutions of differing antibody content, clearly indicating the greater antibody content of fluid B. All three tests indicate that the technique enables the detection of anti-bovine γ-globulin antibody in sera if it is present in a concentration of approximately 30 μg. per ml. Since only 0.02 to 0.04 ml. of serum was used for each tube, the test is quite sensitive, actually detecting the presence of 0.6 to 1.2 μg. of antibody protein in the small test specimens used. Other tests also carried out with solutions of known antibody content showed, like the one here described, that the 1.0 and 0.1 per cent antigen solutions yielded similar end points. Accordingly it seemed possible that either or both of these solutions would serve to estimate the amount of antibody protein that might be present in sera of unknown antibody content, since the greatest dilution of any serum yielding a positive test could be considered to have an antibody protein content of about 30 μg. When using double dilutions of the serum for such a test, the antibody protein content of the undiluted serum was estimated by assuming the next to the last tube to contain 60 μg.—the third from the last to have 120 μg. and so on up to the first tube containing undiluted material.

The technique just described was used throughout the present work employing, however, only the 1.0 and 0.1 per cent solutions of antigen. It is obvious that with this test, sera containing less than approximately 30 μg. of antibody protein (4.8 μg. antibody N) per ml. would be expected to yield negative findings.

Precipitin Tests

Before undertaking the principal experiments as outlined at the beginning of the paper, it was imperative to perform in advance a number of tests to indicate whether the procedures would be expected to yield valid findings. For example, at the outset, one would expect to find only very little antigen in the livers of donor animals after a few weeks. How little antigen could be present and yet elicit the reappearance of circulating precipitins as a secondary response of recipient rabbits treated as described above? The following test yielded a presumptive answer.

The Smallest Quantity of Bovine γ-Globulin Capable of Inducing the Reappearance of Circulating Precipitins in Rabbits Previously Injected with the Antigen.—Work done for other purposes had already shown that rabbits easily withstand three intraperitoneal injections of 15 to 20 gm. of liver tissue. Since, for the experiments to be described below, it was deemed imperative to transfer as much liver—presumably containing antigen—as possible to the prepared recipients a simple test was made to determine how little of the pure antigen, given three times intraperitoneally, would induce the appearance of precipitins in the sera of rabbits treated as follows.

Ten rabbits, weighing 2800 to 3000 gm. were "prepared" by a primary single intravenous injection of 5 mg. of bovine γ-globulin in 5 ml. of 0.9 per cent NaCl solution. These animals were bled 1 month later and repeatedly at irregular intervals thereafter, until precipitin tests showed no antigen or antibody in serum specimens obtained from at least two bleedings. 77 days after their 1st intravenous injection of the antigen, 4 of these rabbits were given 3 intraperitoneal injections of antigen at 48 hour intervals. One received 50 μg. of the protein with each injection, another got 30 μg. in the same way, while the remaining 2 received 20 μg. and 10 μg. respectively with each injection. All the sera of all 4 animals showed positive precipitin reactions for antibody on the 7th day after the 1st injection which was the 3rd day after the last injection, and they continued to show antibody in serum samples taken at various intervals thereafter.

The 6 other prepared rabbits were given, in pairs, only 5 μg., 3 μg., and 2 μg., of the protein, in each of 3 intraperitoneal injections. One of the animals that got the 3 injections of 5 μg. gave a dubiously positive reaction for antibody. All the others yielded negative results.

The conditions for the absorption of antigen in these tests were, of course, optimal, the antigen being in pure solution. The absorption of antigen held within the Kupffer cells of the transferred liver tissue might readily be less until autolysis occurred. There seemed to be no way to test the ability of prepared recipient rabbits to absorb antigen held in this way. The mere addition of free antigen solution to a suspension of liver tissue would not simulate the conditions of the transfer experiments, since others have shown—and we have confirmed the fact—that bovine γ-globulin, in aqueous solution mixed with suspensions of liver tissue, can readily be recovered after centrifugation in the supernatant fluid. In this state its absorption from the peritoneal cavity of a recipient rabbit would presumably be like that of the trials just performed.

It can only be said, from the tests as carried out, that the prepared rabbits gave a secondary response, showing circulating antibody detectable by the precipitin test, to as little as 30 μg. of bovine γ-globulin following its injection, intraperitoneally, in three divided doses. Since some of the work from this laboratory (1–4) and some findings of others (9–20), all of which will be discussed after presenting the data obtained in this work, suggested that one might expect to find much more than 30 μg. of antigen in the livers of rabbits injected, even a few weeks previously,

with large doses of foreign protein, the following experiments were carried out as planned.

FINDINGS

No Detectable Antibody Formed by Recipient Rabbits Given Liver Tissue from Donors Injected with Antigen 4 Weeks Previously.—Liver tissue was obtained as already described from two donor rabbits 28 days after they had been injected with bovine γ-globulin. The tissue was transferred in the usual way to two recipients 55 days after the latter had been given their preparatory injections. Each recipient received material from a single donor only. The sera of these recipients had neither antigen nor antibody. The sera of the donors showed no antigen but they contained 960 and 480 μg. of antibody protein per ml. respectively, as determined by the semiquantitative precipitin tests. Consequently, some antibody was necessarily carried over to the recipients in the 60 gm. of liver that each received. Nevertheless, the sera of both recipients, taken every day for 10 days, and every other day until the 18th day, showed no positive precipitin reactions for either antigen or antibody. There was no trace of antibody either directly absorbed or formed by the recipients.

Since the sera of the donors in this experiment contained antibody, but none appeared in the blood of the recipient rabbits, the finding indicates that the small amount of antibody, present in blood trapped in the donors' liver tissue and transferred directly to the recipients, was too slight to be detectable in the blood of the latter after its absorption from the peritoneal cavity and subsequent dispersion throughout the body. Accordingly, the findings from this experiment suggest three possibilities; that after 4 weeks the donors' liver tissue contained too little antigen to elicit detectable antibody formation in the recipients, or the antigen had become too degraded in this interval of time to form precipitating antibody, or no antigen had persisted in the tissue at all.

Findings Obtained with the Hemagglutination Test.—It seemed wise, next, to determine whether a more sensitive detection test might demonstrate the presence of antibody in the sera of these recipients, and thereby indicate if minute amounts of intact antigen, too little to engender enough antibody to be detected by the precipitin technique, had been present in the transferred liver tissue. This more sensitive test might also show whether traces of directly transferred antibody would appear.

Accordingly, several of the negative samples of sera obtained in the last experiment were subjected to the extremely sensitive hemagglutination test of Boyden (21), using for the purpose serum obtained from that prepared recipient which had received material from the donor having the larger amount of estimated antibody (960 μg. per ml.) in its serum. Two samples of sera, taken 1 week and 2 days, respectively, before the transfers were made—both of which had yielded negative precipitin tests for antibody—showed weakly positive hemagglutination reactions. Suitable controls of normal sera were negative. Following the transfers, samples of sera of this recipient obtained on the 3rd, 7th, and 10th day after the last transfer also showed weakly positive hemagglutination tests for antibody, the titer of which was no greater than that of the specimens taken before the transfers were made. There was, therefore, no evidence of antibody formation by the recipient, nor any evidence for the detection of directly transferred antibody.

Findings with Liver Tissue and a Certain Liver Fraction Taken from Donors 3 Weeks after

Injecting Them with Antigen.—In the next 3 experiments liver tissue was taken from 3 donors only 21 days after they had been injected with antigen. Three recipients received, at 48 hour intervals, 3 transfers containing 20 gm. of tissue in each. Two of these animals were bled at daily intervals for 9 days and at 48 hour intervals thereafter until the 16th day after the 1st transfer. The third animal was bled on the 2nd, 4th, 6th, 8th, and 9th day and daily from the 11th to the 15th day after the 1st transfer.

Neither antigen nor antibody were found by the precipitin tests, indicating, as in the preceding experiment, that antigenic material, if present in the donors' liver tissue at all, was either retained in such small amounts or degraded to such a degree that it could no longer stimulate the formation of antibody in amounts detectable by the precipitin test.

It is of interest, however, that the sera of the donors in this experiment were relatively rich in antibody and showed positive readings by the semiquantitative precipitin test in doubly diluted sera in the 6th or 7th tube indicating antibody protein contents of 960 to 1920 μg. respectively. Here again, as in the preceding experiment, some antibody in the blood contained in the donors' liver tissue must have been transferred to the recipients, yet, either because of a lack of absorption or because of dilution in the bodies of the recipients, none became detectable in the blood.

A final transfer was then made using a certain fraction of liver tissue taken from a 4th donor 22 days after it had been injected with antigen. The fraction was obtained in the following way. In work done for other purposes rabbits had been injected intravenously with a blue tracer antigen formed by coupling a dye to bovine γ-globulin, as described elsewhere (1–3). Following its injection the material appeared mostly in Kupffer cells within the liver. When liver tissue, taken from animals injected with this material was homogenized and ground, as in the present experiments, but then made up to 2 liters in volume with 0.9 per cent NaCl solution and allowed to settle for 2 to 3 hours in 2 separate 1 liter graduates, most of the blue material appeared in a layer of the suspension lying between the 200 and 400 ml. marks on the graduates. After removing this layer much of the blue material could then be recovered by centrifugation at about 1600 R.P.M.

In the present experiment a layer of this sort was obtained from a suspension of the whole liver from the 4th donor that had been injected with bovine γ-globulin 22 days previously. This material was injected, in 3 equal parts at 48 hour intervals, into the peritoneal cavity of a prepared recipient rabbit. All samples of serum obtained from this animal, at daily intervals for 10 days and thereafter every other day until the 16th day, yielded negative precipitin tests for both antigen and antibody.

Antibody Formation Occurred in Recipient Rabbits Given Liver Tissue from Donors Injected Two Weeks Previously with Large Amounts of Bovine γ-globulin.—Liver tissue from 3 donor rabbits, taken 14 days after they had been injected with antigen, was transferred 3 times to each of 3 recipient rabbits, in the manner already described, each recipient receiving tissue from only one of the donors. Two of these recipients had been given the primary injection of antigen 75 days previously, and the third 55 days earlier. Their sera contained neither antigen nor antibody detectable by the precipitin test. Although the sera of the donor rabbits contained no antigen, approximately 240 μg. of antibody protein was present per ml., as judged by the semiquantitative precipitin test. Accordingly, the transfer of liver tissue entailed, as already discussed, the simultaneous transfer of whatever antibody might be left in the blood within the tissue. Since the amount of antibody in the sera of these do-

TABLE II

Table II summarizes the results obtained with qualitative and semi-quantitative precipitin tests, carried out as described in the text, for antigen and antibody in the sera of three prepared, recipient rabbits (A, B, and C). These recipients received 3 transfers each of liver tissue from donor rabbits injected 2 weeks previously with large amounts of bovine γ-globulin as the antigen (see text).

Treatment of recipient rabbits A, B, and C		Day after 1st transfer	Day after 3rd transfer	Qualitative precipitin tests			Semiquantitative precipitin tests for antibody only			
					For antibody		Highest dilution of serum giving a positive reaction against BGG solutions of:		No. of the last tube in the series to have a precipitate	Estimated* μg. of antibody protein per ml. of serum
				For antigen	*vs.* 1 per cent BGG solutions	*vs.* 0.1 per cent BGG solutions	1 per cent	0.1 per cent		
1st transfer to recipients	A	0	—	0	0	0				
	B	0	—	0	0	0				
	C	0	—	0	0	0				
2nd transfer to recipients	A	2	—	0	0	0				
	B	2	—	0	0	0				
	C	2	—	0	0	0				
3rd transfer to recipients	A	4	0	0	0	0				
	B	4	0	0	0	0				
	C	4	0	0	0	0				
	A	6	2	0	0	0				
	B	6	2	0	0	0				
	C	6	2	0	0	0				
	A	7	3	0	wk+*	‡	Undiluted only	Undiluted only	1	30
	B	7	3	0	0	0	0	0		
	C	7	3	0	0	0	0	0		
	A	9	5	0	+	+	1/4	1/4	3	120
	B	8	4	0	wk+	wk+	Undiluted only	Undiluted only	1	30
	C	8	4	0	wk+	wk+	Undiluted only	Undiluted only	1	30
	A	11	7	0	+	+	1/8	1/8	4	240
	B	9	5	0	+	+	1/4	1/4	3	120
	C	9	5	0	wk+	wk+	Undiluted only	Undiluted only	1	30
	A	13	9	0	+	+	1/16	1/16	5	480§
	B	10	6	0	+	+	1/8	1/8	4	240
	C	11	7	0	wk+	wk+	Undiluted only	Undiluted only	1	30
	A									
	B	12	8	0	+	+	1/8	1/8	4	240§
	C	13	9	0	wk+	wk+	Undiluted only	Undiluted only	1	30§

* See text.
‡ Faint trace but definitely positive.
§ Experiment terminated.
wk+, weak positive reaction.
BGG, bovine γ-globulin.

nors was much less than that of the donors in the preceding experiments there was no reason to expect the appearance of directly absorbed antibody in the recipients' blood. Nevertheless, to determine whether antibody would be absorbed from the peritoneal cavities of the recipients in sufficient amounts to become detectable in their sera, the animals were bled at frequent intervals as indicated in Table II which summarizes the findings in the sera of these recipients, as tested by the semiquantitative precipitin technique, against both 1 per cent and 0.1 per cent solutions of bovine γ-globulin. As the table shows, no antigen appeared in the blood of these animals at any time, and no antibody was found in the blood until the 7th day after the 1st transfer of liver, the 3rd day after the last transfer, at which time the faintly positive tests for antibody showed themselves only in the tubes containing undiluted serum of the first recipient (A). There was, therefore, during the first 6 days no evidence of the absorption of transferred antibody.

Nine days after the 1st transfer (on the 5th day after the last transfer) the precipitin reactions obtained with the serum of recipient A were much stronger than at the previous bleeding, the semiquantitative test yielding positive precipitins with the 4-fold dilution of serum, thereby indicating an antibody protein content of 120 μg. per ml. Two days later, as the table shows, the reactions for antibody became stronger, positive in the serum diluted 8 times (240 μg. of antibody protein per ml.) and after another 48 hours positive after a 16-fold dilution (480 μg. of antibody protein per ml.), a result that would not have been found through the simple absorption of transferred antibody. On this day, the experiment was terminated.

The findings with the second recipient, B, were similar to those obtained with recipient A except that antibody appeared later, that is to say, in the blood taken on the 8th day after the 1st, the 4th day after the 3rd, transfer of liver. The following day the precipitin tests were stronger, positive in serum diluted 4 times (120 μg. of antibody per ml.), and on the 10th and 12th day at an 8-fold dilution (240 μg. of antibody protein per ml.). On the 13th day, the animal was found moribund, and the experiment was terminated. Clearly, however, there was no evidence of transferred antibody in this experiment. The increasing amounts of antibody found seemed to have been formed by the recipient. More will be said of this below.

A third similar experiment was made. No antibody appeared in the recipient's serum during the first 7 days. On the 8th to the 13th day, when the experiment was terminated, weakly positive reactions appeared with the undiluted serum only, and not with serum dilutions. Very little, if any, antibody was present.

Obviously in these trials some circulating antibody of the donors must have been transferred in the blood trapped in the liver tissue that had been injected into the recipients. Since, however, antibody did not appear in the blood of the recipients until the 7th or 8th day after the 1st transfer (the 3rd and 4th day after the last transfer) it seemed unlikely, though possible, that after such a long interval it could be merely transferred antibody. The fact that in 2 of the tests antibody continued to increase after its first appearance, until the end of the experiments, indicated that it was being formed in the recipients rather than being absorbed by them. Passively absorbed antibody would have appeared sooner and disappeared more rapidly. Further, it is to be noted that the circulating antibody in 2 of the recipients, as found by the semiquantitative tests, was approximately as strong as that in the donors from which the liver tissue was derived. To obtain so much antibody in the circulation of the recipient by absorption it would have been necessary to transfer all the blood of the donor, not merely the blood in 60 gm. of liver tissue. It may be recalled that

in the experiments described earlier, in which liver tissue was taken from donors 3 and 4 weeks after they had been injected with antigen, there was far more antibody in the blood, at this time interval, than in the blood of the donors killed 14 days after the injection of antigen. Nevertheless, after transferring liver tissue, from these animals, with the higher blood antibody content, no antibody was ever detected in the recipients.

The data obtained in this experiment indicate that the recipient rabbits formed circulating, precipitating antibody to the antigen transferred to them in the donors' liver tissue, antigen which had persisted in that organ for 2 weeks after the donors had been injected.

DISCUSSION

The findings, here reported, lend themselves to several interpretations. At some time, probably close to the end of the 2nd week after injecting the donor rabbits with bovine γ-globulin, the antigenic material in their livers may have become completely destroyed or eliminated, or so reduced in amount that even 60 gm. of tissue, when transferred to the recipients, contained too little intact antigen to engender detectable amounts of circulating antibody in these animals. On the other hand, although each of these processes undoubtedly took place to a greater or lesser degree, some of the antigen, or the antigenic material, in the liver tissue may have suffered a different fate, becoming degraded but not totally destroyed or eliminated, so that, after 2 weeks, some kind of antigenic breakdown products persisted in the liver, that were not sufficiently like the original antigen to call forth antibodies which could yield precipitates with solutions of bovine γ-globulin. Is there a choice to be made among these interpretations?

As already mentioned, workers in several laboratories (9–20) have injected rabbits, rats, and mice with foreign protein antigens, among them bovine γ-globulin, coupled with radioactive labels. Some of these authors have indicated that the labels, especially C^{14}, S^{35}, and to a lesser extent I^{131} remain largely coupled to the injected foreign proteins after their introduction into the body. Nevertheless it is well recognized, of course, that the measurement of residual radioactivity coupled to the protein, as injected, does not necessarily indicate whether the protein portion of the injected antigen has been left intact or considerably broken down. In view of the likelihood of the latter possibility a consideration of some of the data obtained with these radioactive antigens throws much light upon the condition or state of the antigenic material dealt with in our present experiments.

Crampton, Reller, and Haurowitz (9), after injecting C^{14} anthranilazo-ovalbumin into rabbits, found after approximately 1 day, 12, and 32 days, 1.35, 0.50, and 0.185 per cent, respectively, of the injected activity still in the liver. Friedberg, Walter, and Haurowitz (10), after injecting rabbit, guinea pig, and chicken serum albumin and serum globulin doubly labelled with I^{131} and S^{35} into rats, found about 0.6 per cent of the injected activity in the livers after as long as 45 days. The radiosulfur persisted longer than the radioiodine. They concluded that the high percentage of S^{35} found in the liver protein was not caused by the incorporation of free S^{35} amino acids from the general body pool, and that the C^{14} and S^{35}

labels give a better index of the behavior of radioactive antigenic proteins than the I^{131} label (9, 10); indeed the heterologous protein deposited in the organ, if it was represented by the S^{35} found in the liver proteins, might be 40 to 70 times higher than would be indicated by the I^{131} label.

The Nature of Antigenic Material Retained in the Liver after More Than 2 Weeks. Evidence That it is Degraded but Not Entirely Destroyed.—It is an undisputed truism that antigenic proteins are destroyed sooner or later in the body, and that, for this purpose, the proteins must first be degraded. The matter of interest for the present discussion is: How soon does the antigen first lose its characteristic properties, and what properties remain? The findings of several authors, who have followed the fate of radioactive tracers of labelled proteins, throw some light upon the nature of the material of this sort at various time intervals after its injection into animals.

For example, Ingraham (13, 14) injected mice with a sulfanilic acid–azo–bovine γ-globulin labelled with S^{35}. After showing that the S^{35} found in the tissues many days later (13) was not split off from the compound and bound to tissue proteins, he demonstrated that the disappearance of the S^{35} label of his antigen took place in 2 stages. The first was rapid, lasting about 15 days and having a half-life of 1.6 days. The second, with a half-life of 15 days, was much slower and endured far longer. Data from the tables in one of his papers (13) indicate that 20 per cent or more of the radioactivity in the liver, found at 24 hours, remained after 15 days, that 1 to 4 per cent might remain for as long as 200 days. Fig. 3, in his paper (13) indicates that, by the 22nd and 30th day, there was present in the liver about 4 to 5 per cent of the initial activity found after 24 hours. It is of some interest that the interval of time, 15 days, after which the rapid rate of loss changed to the slow rate, is the same as that during which, in the experiments discussed here, transferred liver tissue was found capable of eliciting the formation of precipitins in the recipient animals. Moreover, the length of the period of slow decline in Ingraham's experiments corresponds to the length of the period during which liver tissue, transferred from rabbits injected with antigen (2–4), produces anaphylactic sensitivity in recipient mice. Ingraham obtained definite evidence for the degradation of his sulfanilic acid–azo–bovine γ-globulin labelled with S^{35}. Supernatant fluids from suspensions of liver, taken 17 days after injecting the antigen, showed that a large proportion of the S^{35} activity was bound to material which would not sediment at 18,000 g in the centrifuge, but which was large enough to resist dialysis through Visking tubing. This material would not precipitate with anti-bovine γ-globulin serum.

Garvey and Campbell (16, 17) describe changes in a soluble protein antigen, hemocyanin-*p*-azo-phenylsulfonate labelled with S^{35}, taken up by the liver after its injection into the blood stream of rabbits. Three days later, both precipitating and non-precipitating fragments were isolated from the tissue as shown by electrophoresis, salt precipitations, and specific inhibition tests. Twenty to 30 days after an injection of 30 mg. of the antigen, approximately 1 per cent of the radioactivity was still present in the liver, about 300 μg., judging from the curves presented (17). Five to 6 weeks after injection only non-precipitating material remained.

It is to be stressed that only a very short persistence of intact antigen has been reported by Coons and his coworkers (22–24), using fluorescein-conjugated antibody to detect histologically the possible sites of antigen retention. Dixon and his collaborators (25–27), working with the I^{131} label, find no evidence for antigen retention in

the blood after antibody formation becomes well established, nor do they find evidence for its long retention in the tissues. They point out (25) that the decline of antibody formation 7 or 8 days after an injection of antigen indicates the loss of the effective antigenic stimulus for serologically demonstrable antibody formation.

The data of the present paper tend to agree with this latter view inasmuch as the antigen taken up by the livers of the donor rabbits appeared to be degraded, since, after 2 weeks, it was not sufficiently intact to elicit the formation of precipitins in the recipients. Some of the work carried out with radioactively labelled protein antigens, especially that of Ingraham (13, 14) and Garvey and Campbell (16, 17), also indicates, as already discussed, a slow degradation of injected antigen rather than its rapid, total elimination, since much of the retained radioactivity seems to be coupled to changed antigenic material. Under these circumstances the radioactive labels of injected protein antigens do not serve as reliable indicators of intact, retained antigen.

The relatively short survival of injected antigen, in its intact form, as indicated by the present findings and in the reports of some of the authors mentioned above (22–27), stands at variance with observations obtained in previous work from this laboratory (2–4). In that work, as already stressed, bovine γ-globulin, injected into donor rabbits as an antigen, seemed to persist in their livers for 6 weeks or more, as judged by the fact that transfers of the donors' liver tissue after these periods rendered recipient mice anaphylactically sensitive to the antigen given to the donors (4). These divergent views could be reconciled if it could be shown that antigen, as it is destroyed, can pass through stages of degradation in which it loses its capacity to engender precipitating (complete) antibodies in recipient animals while it can still sensitize them anaphylactically.

Still another explanation for the findings described in the present paper is suggested by the work of Sternberger and his colleagues (18–20), who gave both single and multiple injections of bovine γ-globulin to rabbits, using 100 mg. per kilo of body weight—the same dose that was given to the donor rabbits in the present experiments. Two days after a reinjection of the protein the free antigen disappeared from the blood. By the 4th day however, an antigen-antibody complex appeared in the circulation. The antigen must have come from the tissues since there was none present in the serum 2 days earlier. The authors believe that the antigen may have undergone some metabolic changes, and they cite unpublished data which indicate that the resulting antigen-like material may have been present in the blood together with detectable antibody, either as a breakdown product of the original antigen or as a new material furnished by the host. They state that these breakdown products reacted with non-precipitating antibody. That products of this sort might exert some sort of antigenic activity is no longer beyond the bounds of reason since recent work by Stahmann, Tsuyuki, Weinke, Lapresle, and Grabar (28) has demon-

strated the antigenicity of certain incomplete proteins, polypeptides, and peptides. It could therefore be possible that bovine γ-globulin, injected into the donor rabbits of the present work, could be degraded after 2 weeks to such an extent that only antigenic material of this sort remained, incapable of engendering precipitin formation in the recipients but able to render mice anaphylactic to the original antigen, and capable, if this bovine γ-globulin had been labelled with S^{35}, C^{14}, or even with I^{131}, of retaining some of the label.

Finally one more point. In spite of the brief retention of antigen in the livers of the donor rabbits of these experiments—at least in a state sufficiently unchanged to elicit the formation of precipitins in the recipient rabbits—the antigen may have persisted somewhat longer than we have found. Experiments of the sort outlined here could not be expected to detect extraordinarily minute amounts of retained antigen, and negative results would be expected to occur before the last antigen had disappeared. However, since precipitin tests showed positive results when liver was taken from the donors 2 weeks after injecting them, but the far more sensitive hemagglutinin test yielded negative findings after 3 weeks, one can be justified in suspecting that degradation of the antigen had progressed considerably by the 3rd week.

SUMMARY

The findings presented in this paper indicate that 60 gm. of liver tissue, taken from adult donor rabbits 2 weeks after injecting them with large amounts of bovine γ-globulin, and transferred to the peritoneal cavities of recipient rabbits prepared for a secondary reaction, contain enough of the antigen to induce the formation of detectable amounts of precipitin in the recipient rabbits. On the other hand, liver taken from donors 21 and 28 days after injecting them with the same antigen fails to bring about a similar effect. The findings outlined in the paper suggest that the antigen is degraded after the 2nd week to such an extent that it fails to engender the formation of complete antibody in the recipient animals, although previous work has shown (2–4) that the transfer of liver tissue from similar donor rabbits to mice renders the recipient mice anaphylactically sensitive to the antigen, bovine γ-globulin.

The implications of the findings are discussed.

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