II. ANTIGENICITY OF GELATIN IN RABBITS AND OTHER SPECIES*, ‡

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In the preceding publication (1) the presence of naturally occurring antibodies to gelatin in man was demonstrated. In view of these findings it was decided to analyze the sera of other species for naturally occurring antibodies to gelatin. In addition attempts were made to immunize rabbits employing various techniques. The results of these investigations will be the subject of this second paper.

EXPERIMENTAL

Antigens.—Gelatin. Gelatin Lot No. P 107-20, furnished by Dr. D. Tourtellotte of the Knox Gelatine Company, was the source of the gelatin (beef bone) used both in the analyses of the many different sera and for the immunization of the rabbits. It was supplied as a sterile 6 per cent solution and was the "clinical" type of gelatin. It was similar to the stage B preparation characterized by Scatchard *et al.* (2). The average molecular weight of this material was 36,000.

Oxypolygelatin (OPG). The OPG used in the immunization of rabbits was that referred to in the preceding paper as clinical OPG (Baxter S 230 X).

Nitrogen analyses of the gelatin solutions were performed by the Markham modification of the micro Kjeldahl procedure (3). All antigen solutions were adjusted to pH 7.5, and preserved with 0.01 per cent merthiolate.

Immunization of Rabbits.—(*a*) OPG. Six healthy male albino rabbits were bled and then given a full course of immunization (16 injections) with alum-precipitated OPG during 1 month. 1 week after the last injection of OPG the rabbits were bled by cardiac puncture.

(*b*) Gelatin. The immunization of another group of five rabbits was performed using the gelatin incorporated in a water-oil adjuvant mixture. The emulsions were prepared in a Waring blender with 10 parts of 6 per cent gelatin solution, 1 part arlacel C (4) and 9 parts bayol F (5). For the first course of immunization the animals received three injections per week for 3 weeks of 1.0 ml. of the adjuvant mixture containing gelatin (30 mg./ml.). Dead tubercle bacilli were omitted from this first antigen mixture. The rabbits were bled before immunization and 1, 2, and 3 weeks after the last injection. 3 months later the two surviving rabbits

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were bled and then given another course of immunization similar to above. However, this time the adjuvant mixture contained dead tubercle bacilli. The animals were bled 1, 2, and 3 weeks after immunization. Although rabbit 13-36 was given a third course of immunization the results are not presented here.

"Normal" Sera.—Sera were obtained from the many sources listed below.

Rabbit sera were obtained from normal rabbits and from others which had been immunized with bovine serum albumin, bovine gamma globulin, and ovalbumin.

Horse sera were obtained from the Microbiological Associates, Bethesda, Maryland (horse serum pools 1 and 3) and from Dr. H. Salk of Shalom Farms, Mars, Pennsylvania (horse 2).

Pig sera, sheep sera, and bovine sera were collected at a local slaughter house (Fried and Reineman Packing Company).

Guinea pig sera was obtained fresh from our own laboratory stock and in the lyophilized form from the Texas Biological Laboratory, Fort Worth, Texas.

The serum from rats, dogs, cats, and monkeys was obtained from different laboratories at the University of Pittsburgh School of Medicine, School of Dentistry, and School of Public Health.

Dr. Alan Boyden of the Rutgers University Serological Museum supplied the sera from crab, blue marlin, and blue shark.

All serum samples were handled with sterile precautions. In addition merthiolate to a concentration of 0.01 per cent and phenol to 0.25 per cent were added.

Quantitative Precipitin Studies.—In the original survey of sera from the many species for antibodies to gelatin 3.0 ml. and in some cases 4.0 ml. of clarified serum were measured into 8 ml. centrifuge tubes calibrated at the 2.5 ml. mark. Increasing amounts of gelatin (3, 6, 10, 16 gammas N) were added to successive tubes, and the same procedure for analysis employed as in the previous paper (1). After it was determined that some normal sera had considerable amounts of antibody precipitated by gelatin varying quantities of serum were employed for other analyses.

The pre- and postimmunization sera from the rabbits were assayed for antibody N by the quantitative precipitin method of Heidelberger and MacPherson as described in the previous paper. The Folin-Ciocalteu tyrosine reagent was standardized against known amounts of rabbit gamma globulin (fraction II) obtained by refractionating Armour fractions II and III, Lot 349-90 according to reference 6.

In order to convert the optical density at 7500 A produced by the reaction of the Folin-Ciocalteu reagent with precipitates from different species, it was necessary to prepare gamma globulin from these species as standards. In the cases of pig, guinea pig, monkey, rat, and cat sera the gamma globulin was prepared by the method described in reference 7. Canine gamma globulin prepared by Armour and Company served as standards for the dog antibody. The antibody precipitated from a horse antipneumococcal polysaccharide S XIV by the homologous antigen was used in obtaining horse gamma globulin standards.[1]

Hydroxyproline Analyses.—Qualitative tests for hydroxyproline were made with precipitates from different species to ascertain the presence of gelatin in the precipitates. The method of Neuman and Logan was employed (8).

Solubility of Specific Precipitates.—As described in the previous paper gelatin-antigelatin precipitates were formed and their solubility in 1.0 ml. of different protein solutions containing 1 mgN./ml. was determined.

Resuspension of Specific Precipitates (Dog) in Rabbit Anticanine Gamma Globulin Sera.—Gelatin-antigelatin precipitates from dog serum were resuspended in rabbit anticanine gamma

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[1] We wish to thank Dr. M. Heidelberger for making available both the horse antisera and pneumococcal polysaccharide.

globulin. The method of analysis and the procedures used were the same as given in the previous paper.

Passive Transfer (Anaphylaxis) Studies.—2 ml. of horse serum containing about 20 μg. of antibody N (antigelatin) per ml. was injected into guinea pigs, followed 48 hours by an injection of 1 mg. of gelatin N.

Ultraviolet Spectra.—The ultraviolet absorption spectra of specific precipitates from dog, horse, and rabbit were compared with the standards mentioned above.

RESULTS

Table I summarizes the results obtained on the many different species as to the presence or absence of naturally occurring antibodies to gelatin. As mentioned in the previous paper quantities of antibody nitrogen below 2 μg. per ml. of serum are not considered significant.

TABLE I

Species Analyzed for Antibodies to Gelatin in Normal Sera

Antibodies present		Antibodies absent	
Horse	Guinea pig	Chicken	Crab
Dog	Cat	Cow	Shark
Pig	Rat	Sheep	Marlin
Monkey		Rabbit	

The types of precipitin curves obtained with the various normal sera are shown in Figs. 1 and 2. Although the complete curves are not shown for all the sera studied, data were obtained which indicated inhibition of precipitation when excess gelatin was added to the sera. These complete curves will appear in subsequent publications when various gelatins and derivatives of gelatin will be compared immunochemically.

Attempts to immunize rabbits with alum-precipitated OPG were futile. Table II presents the data in the five rabbits of the first and second course immunizations with gelatin incorporated in an adjuvant mixture. Of the five rabbits that underwent the first course immunization three showed significant increases in antibody precipitated by gelatin. One of the non-reactors (1354) responded well to the second course. The week to week variation in antibody level will be discussed later. None of the rabbits immunized with alum-precipitated OPG produced detectable amounts of antibody. Their sera were analyzed both with OPG and gelatin with exactly the same negative results.

The data presented in Table III indicate that the gelatin-antigelatin precipitates obtained in the many different species were soluble only in excess gelatin which is one of the criteria of a true antigen-antibody reaction (9). The protein solutions which served as suspending media were the same as those used in the first publication.

That the proteins precipitated by gelatin resembled the gamma globulin was indicative from the ultraviolet absorption spectra of the dissolved precipitates. Further evidence of this fact is presented in Table IV. Resuspension

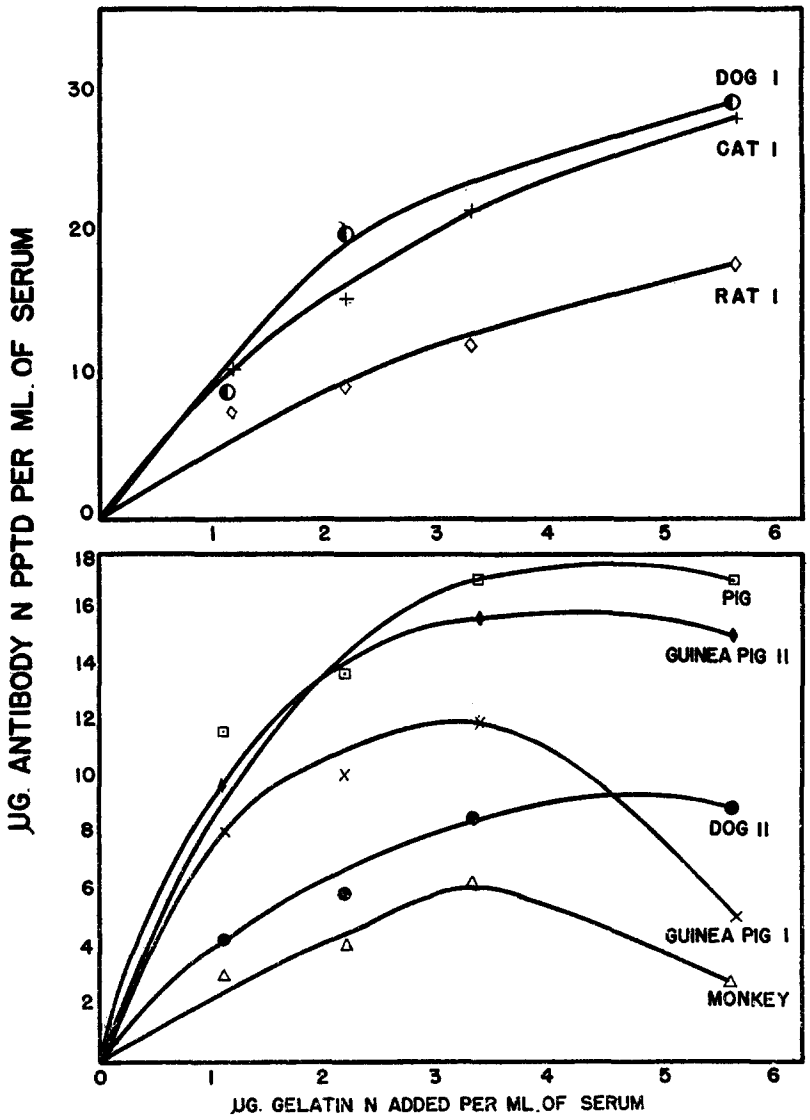

FIG. 1. Precipitin curves of horse sera.

of precipitates from dog serum in rabbit anticanine gamma globulin serum caused the precipitates to pick up significantly more protein material (anti-CGG) than the same precipitates resuspended in normal rabbit serum.

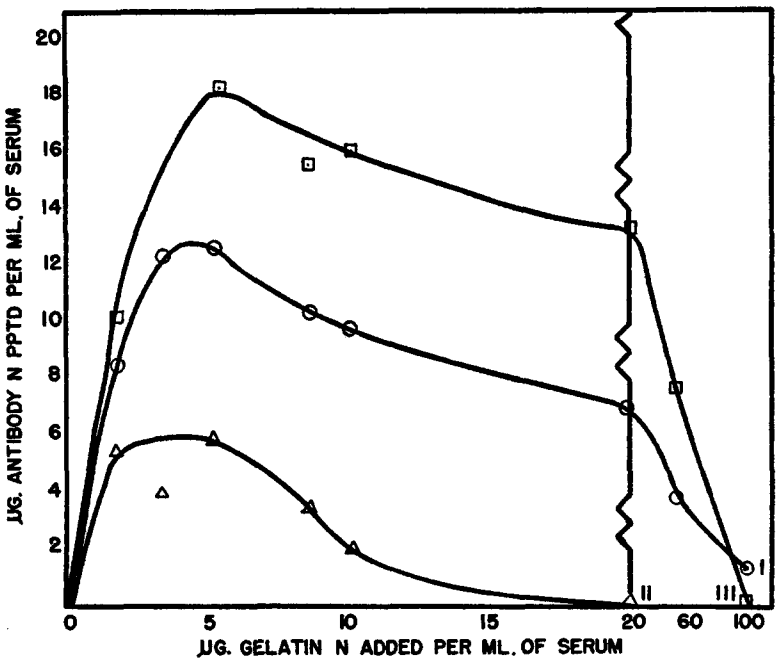

FIG. 2. Precipitin curves of various sera.

TABLE II

Response of Rabbits to Immunization with Gelatin-(Knox P-20)

Rabbit No.	Bleeding	First course			Second course	
		μg. gelatin N added per ml. serum				
		1.1	3.4	5.7	1.1	3.4
		μg. antibody N precipitated per ml. serum				
13-34	Preimmunization	0.7	1.5			
	W 1	5.7	7.3			
	W 2	3.8	3.8	3.2		
	W 3	3.5	4.0			
13-35	Preimmunization	0.0	0.3			
	W 1	0.8	0.7	0.0		
	W 2	0.5	1.1	1.9		
	W 3	0.4	0.9	0.5		
13-36	Preimmunization	0.0	1.8		0.6	—
	W 1	8.5	12.5		4.0	3.0
	W 2	5.1	5.1	3.2	4.6	4.0
	W 3	1.7	0.8	0.8	7.2	6.5
13-37	Preimmunization	0.5	—	—		
	W 1	4.3	3.8	2.7		
	W 2	2.4	2.7	2.0		
13-54	Preimmunization	1.8	—	—		
	W 1	0.6	1.0	0.8	1.1	1.5
	W 2	0.9	1.5	0.9	5.4	4.9
	W 3	1.5	1.2	1.5	6.6	6.6

In the previous publication mention was made of the fact that with human sera the gelatin-antigelatin system did not fix complement N. As it is known that guinea pig serum has considerably more complement than human serum, frozen guinea pig serum was studied before and after decomplementation.

TABLE III

Solubility of Gelatin-Antigelatin Precipitates

Diluent	Species			
	Horse	Dog	Pig	Monkey
	Optical density 7500 A			
Gelatin, *1 mg. N/ml*	0.030	0.040	0.032	0.033
Gel-CH_2O, *1 mg. N/ml*			0.068	
Gel-MFG, *1 mg. N/ml*	0.210	0.255	0.340	
BSA, *1 mg. N/ml*	0.300	0.293	0.390	0.150
Ea, *1 mg. N/ml*	0.350	0.350		0.160
0.9 per cent NaCl	0.340	0.350	0.400	0.165

TABLE IV

Optical Density at 7500 A of Gelatin-Antigelatin Precipitates (Dog) Resuspended in

Normal rabbit serum	Rabbit anti-CGG serum
0.063	0.136
0.110	0.205
0.119	0.246
0.138	0.265

TABLE V

Effect of Decomplementation of Guinea Pig Serum on Gelatin-Antigelatin Reaction

μg. gelatin N added per ml. serum	μg. antibody N precipitated per ml. serum	
	Untreated	Decomplemented
1.1	8.7	7.8
3.4	11.5	10.8
6.7	10.6	10.2
13.3	11.0	9.9

100 μg. N of an egg albumin–rabbit antiegg albumin specific precipitate was used per ml. of guinea pig serum (10). Table V shows no significant difference between the decomplemented and undecomplemented sera. As mentioned in the first communication Watson *et al.* (11 *a*) and Loiseleur and Urbain (11 *b*) have detected antibodies to soluble rat collagen in rabbits. They detected anti-

bodies to collagen employing complement fixation techniques. It is quite possible that the collagen system fixes complement whereas the gelatin one does not. None of the guinea pigs given horse serum containing antibody to gelatin exhibited signs of anaphylaxis upon injection of gelatin. However, upon injection of anti-BSA followed by BSA all the guinea pigs died.

DISCUSSION

After it was found that there are antibodies to gelatin in most human sera it was thought that an analysis of the sera from different species may give some insight into the source of these antibodies. As discussed previously the only two suggestions as to source are either that the antibodies are produced in response to a dietary source of gelatin or are produced as part of the mechanism involved in the metabolism of collagen. The results of the findings presented here do not definitely favor either mechanism. It is reasonable to attribute the absence of antibodies to gelatin in the sera of chicken, cow, sheep, and rabbit, and their presence in dog, pig, monkey, cat, and rat sera to a dietary source. The absence of antibodies to gelatin in the sera from crab and fish cannot be explained on a dietary basis since it is known that crab and fish do eat meat or other fish. Both the guinea pigs and rabbits in our laboratory were kept on diets which according to the manufacturer had no gelatin source. It then becomes difficult to explain the presence of antibodies in guinea pig sera as arising from a dietary source. The results with the horse sera too are difficult to interpret. Although it has not been possible so far to determine whether the diets of the horses contained meat base or not, it is generally considered that the horses would be herbiverous.

If the antibodies to gelatin are related to the metabolism of collagen one would then have to assume that the metabolism of collagen in various species is different. This of course is not too difficult to accept.

It is contemplated to place some experimental animals, such as rat, cat, and dog, on diets which do not contain any beef bone or other gelatin source, bleed them after a few months and then change the diet to one which has a high content of gelatin. After a period of time the animals will be bled again to see whether or not the diet had any effect on the level of antibody to gelatin. As mentioned in the first paper it is also hoped to obtain sera from true vegetarians to see whether or not they have significantly lower levels of antibody to gelatin.

It was not possible to immunize rabbits with alum-precipitated OPG. However, the original gelatin incorporated into the H_2O in oil adjuvant mixture was successful in inducing the formation of antibodies. The level of antibody produced was not as high as is usually observed when adjuvants are employed. Moreover the antibody level was not as prolonged as has usually been observed. The animals that responded to the first course of immunization had the highest

concentration of antibody at the end of the 1st week. This usually dropped by the 2nd week. The reimmunization of the two surviving rabbits 2 months after their last bleeding resulted in increased antibody formation. However, the level of the antibody response did not resemble a true anamnestic response, although here the concentration of antibody was fairly level for 2 to 3 weeks. Immunization of rabbits with modified fluid gelatin incorporated in a water-in-oil adjuvant led to the formation of a high level of antibodies which has persisted for at least 2 months (12).

Possible explanations for these observations may be (*a*) that we are dealing with a denatured protein and therefore it is not markedly antigenic; (*b*) the adjuvant mixture may not have been too stable and the efficacy of the emulsion technique for immunization was diminished. Watson *et al.* (11 *a*) experienced considerable difficulty in immunizing their rabbits with soluble collagen. Only after 3 to 6 months of immunization (intraperitoneal injections) were antisera of satisfactory titer obtained.

The data on the solubility of the specific precipitates, the ultraviolet absorption spectra of the precipitate, and the reaction of precipitates from dog serum with anticanine gamma globulin sera indicate that here too we are dealing with a true antigen (gelatin)-antibody reaction. The observation that neither human serum (1) nor horse serum containing antibodies to gelatin could sensitize the guinea pigs might be explained on the basis that the guinea pigs had antibody to gelatin to begin with.

SUMMARY

Normally occurring antibodies to gelatin have been demonstrated in sera from many species.

Immunization of rabbits with gelatin has been successful when adjuvant techniques were employed.

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