I. ANTIGENICITY OF OXYPOLYGELATIN AND GELATIN IN MAN*, ‡

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As there are reports in the literature (1 *a–d*, 2) that highly purified gelatin is non-antigenic, this substance and some of its chemical derivatives have been suggested and tested as possible blood plasma expanders (3). One of the gelatin derivatives which has been proposed is oxypolygelatin (OPG)[1] (4). The recent observations of Kabat and Berg (5) and Maurer (6) that dextran, another plasma expander, although non-antigenic in the rabbit, is a good antigen in man led to the testing of the antigenicity of OPG in man. The results presented in this paper show that gelatin and its derivatives indeed are antigenic in man. The quantitative immunochemical data used to support this thesis and some of the implications of these findings in man will be the subject of this first paper.

EXPERIMENTAL

Antigens.—Oxypolygelatins (OPG). The clinical OPG tested was kindly furnished by Dr. Arthur Cherkin of Don Baxter, Incorporated, of Pasadena. It was Lot No. S 230 X and had an average molecular weight of about 27,000. The molecular weight distribution as determined by Dr. J. W. Williams was as follows: 10,000, 16 per cent; 10,000–20,000, 33 per cent; 20,000–40,000, 33 per cent; 40,000–60,000, 12 per cent; 60,000–80,000, 4 per cent; 80,000–100,000, 1 per cent; > 100,000, 1 per cent.

The "high molecular weight" fraction of OPG S 230 X was obtained by precipitation with two volumes of methanol. This preparation had an average molecular weight of 36,000, based upon correlation of viscosity and osmotic pressure measurements.

Gelatin Lot No. 2160 was also obtained from Don Baxter, Incorporated. This was the material used to prepare the above OPG preparations. It had a number average molecular weight of 50,000–75,000. All of the above materials were found to be non-pyrogenic and non-antigenic in the guinea pig.

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[1] OPG stands for oxypolygelatin.

Nitrogen analyses of the various protein antigens were performed by the Markham modification of the micro Kjeldhal procedure (7). All antigen solutions were adjusted to pH 7.5 and preserved with 1/10,000 merthiolate.

Immunization Procedure.—Healthy medical volunteers were used. An initial preimmunization sample of 50 ml. of blood was taken. Each subject then received two intracutaneous tests with about 0.01 to 0.02 ml. of the OPG or gelatin preparation used for immunization containing 0.25 per cent phenol and a control of 0.85 per cent saline and phenol. After 15 minutes

TABLE I

Response of Humans to Injections of Clinical Oxypolygelatin S 230 X

Subject	No.	OPG solution injected (9 injections)	Preinjection		3 weeks post injection	
			μg. antibody N precipitated per ml. of serum from 3 ml. serum treated with			
			3 μg. OPG N	9 μg. OPG N	3 μg. OPG N	9 μg. OPG N
		mg./ml.				
DAV	1	2	0.0	0.5	0.4	4.4
DJF	2	2	1.0	2.0	—	12.2
CAB	3	2	0.2	1.0	1.5	4.6
JZ	4	2	1.3	2.7	2.0	6.0
JAC	5	2	0.3	0.8	2.2	3.9
DGC	6	5	1.8	3.3	3.3	7.5
JHA	7	5	0.8	3.4	0.8	2.9
NS	8	5	0.7	3.1	1.9	7.5
HJ	9	5	1.1	6.7	1.9	4.7
PHM	10	5	0.0	0.0	2.2	3.6
LH	11	10	0.1	1.3	1.5	2.7
TCH	12	10	0.8	3.1	2.3	4.3
WJH	13	10	0.6	6.4	—	10.3
RNL	14	10	0.0	3.4	0.8	3.3
CHL	15	10	0.6	3.6	1.4	—
SW	16	25	0.8	2.0	0.0	—
RAP	17	25	0.8	3.1	0.1	3.7
TBL	18	25	0.5	—	3.6	—
RRB	19	25	1.1	4.0	3.5	9.4
JRL	20	25	0.2	0.2	—	—

the skin reactions were read. For the initial study with OPG the volunteers were divided into four groups. Each group received nine daily injections of clinical OPG containing 2, 5, 10, or 25 mg. protein per ml. of solution. 3 weeks after the last injection a second 50 ml. sample of blood was obtained and intracutaneous tests performed. Half liter portions of blood were obtained from those individuals who showed good antibody responses.

For the repeat experiment additional medical volunteers were divided into three groups of seven per group. The groups received nine daily injections containing 25 mg. per injection of either OPG S 230 X, high molecular weight OPG or gelatin. Post-immunization bleedings were taken 3 and 7 weeks after the last injection. 4 months later the best antibody producers in each group were reinjected twice with 10 mg. of the appropriate antigen. 3 weeks after the last injection 500 ml. portions of blood were again obtained. All serum samples were

handled with sterile precautions, and in addition merthiolate to a concentration of 0.01 per cent and phenol to 0.25 per cent added.

Quantitative Precipitin Studies.—The pre- and postimmunization sera were assayed for antibody N by the quantitative precipitin method of Heidelberger and MacPherson (8) using the Folin-Ciocalteu tyrosine reagent (9), standardized against known amounts of normal human gamma globulin (fractions II).[2] The complete method is repeated here. After remaining in the icebox for about 10 days, each serum sample was centrifuged in the cold overnight, the lipid removed from the top, the serum decanted, and recentrifuged until perfectly clear before use. In the initial studies with clinical OPG 3.0 ml. portions of pre- and

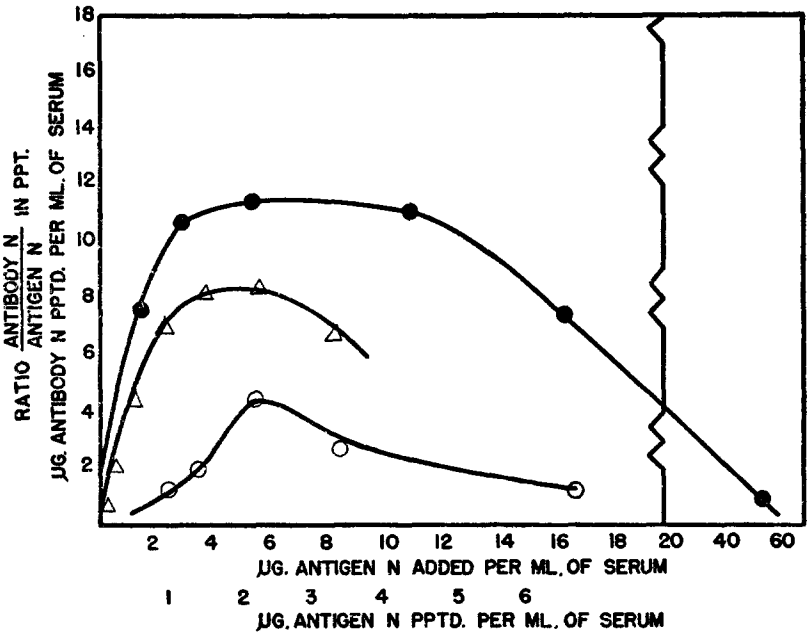

FIG. 1. Calibration curves of human antiserum (RRB.). ○, clinical OPG; △, fractionated OPG; ●, pigskin gelatin.

postimmunization sera were measured into 8 ml. centrifuge tubes calibrated at the 2.5 ml. mark. 3 and 9 μg. of OPG nitrogen in a volume of 1.0 ml. were added to successive tubes. The addition of 1.0 ml. of saline to 3.0 ml. of serum served as the serum control. The tubes were mixed, capped, placed in a water bath at 37°C. for 1 hour and in the refrigerator for from 10 to 14 days with mixing of the tubes daily. The tubes were centrifuged in the cold and the precipitates were washed three times with chilled saline. The dissolution of the precipitates with 0.5 M NaOH at 37°C. was very time consuming. It was later observed that warming the precipitates to 50°C. resulted in immediate solution. This was in accordance with some of the known properties of gelatin which, as will be shown later, was in the precipitate; namely, to dissolve readily upon warming to about 50°C. The dissolved precipitates were made up to the 2.5 ml. mark and 2.0 ml. aliquots analyzed by the Folin-Ciocalteu method at 7500 A using a Beckman model DU spectrophotometer. (Table I).

[2] The author wishes to thank Dr. E. A. Kabat for supplying him with this preparation.

TABLE II

*Response of Human Volunteers to Injection of Oxypolygelatin S 230 X (Group 1)**

Subject	No.	Preimmunization absorption						3 wk. postimmunization absorption‡		7 wk. postimmunization absorption			
		1st		2nd		3rd				1st		2nd§	
		Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen added N per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum
		μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
RWH	21	1.1 OPG	1.0	5.6 OPG	2.7	1.0 gel	1.9	3.9 OPG	1.6	1.1 OPG	5.4	1.0 gel	7.2
		3.3 "	3.8	—	—	1.5 "	6.5	3.0	5.8*	3.4 "	13.4	1.0 "	3.1
		4.1 gel	14.7‖	3.0 gel	4.8					2.0 gel	19.5	2.0 HOPG	2.0
										3.1 "	22.2	2.0 "	0.0
AK	22	1.1 OPG	0.7	5.6 OPG	6.3	1.0 gel	1.9	3.9 OPG	0.0	1.1 OPG	1.8	1.0 gel	0.0
		3.3 "	5.2	—	—	1.5 "	6.5	3.0 gel	3.2	3.4 "	4.3	1.0 "	0.0
		4.1 gel	11.5‖	—						2.0 gel	7.6	2.0 HOPG	0.0
										3.1 "	7.2	2.0 "	0.0
HM	23	1.1 OPG	1.2	5.6 OPG	3.3	1.0 gel	3.7	1.1 OPG	0.0	1.1 OPG	4.6	1.0 gel	5.3
		3.3 "	4.5	—	—	1.5 "	6.6	4.2 "	3.0	3.4 "	10.7	1.0 "	3.5
		4.1 gel	10.0‖	3.0 gel	4.3			3.0 gel	4.8	2.0 gel	19.0	2.0 HOPG	0.0
										4.1 "	17.8	2.0 "	0.0
HH	24	1.1 OPG	—	5.6 OPG	1.0	1.0 gel	3.0	4.2 OPG	0.0	1.1 OPG	2.2	1.0 gel	3.6
		3.3 "	3.0			1.5 "	5.0	3.0 gel	4.0	3.4 "	6.7	1.0 "	2.4
		4.1 gel	8.8‖	3.0 gel	6.1					2.0 gel	11.8	2.0 HOPG	0.0
										4.1 "	12.8	2.0 "	0.0
TH	25	1.1 OPG	0.5	5.6 OPG	0.4	1.0 gel	1.6	1.1 OPG	0.0	1.1 OPG	2.0	1.0 gel	2.2
		3.3 "	1.4			1.5 "	7.7	4.2 "	0.8	3.4 "	3.3	1.0 "	0.7
		4.1 gel	6.9‖	3.0 gel	3.7			3.0 gel	2.9	2.0 gel	9.0	2.0 HOPG	0.0
										4.1 "	6.3	2.0 "	0.0
JB	26	1.1 OPG	0.7	5.6 OPG	1.6	1.0 gel	2.1	1.1 OPG	1.8	1.1 OPG	3.3	1.0 gel	3.7
		3.3 "	2.1			1.5 "	3.9	4.2 "	4.3	3.4 "	6.6	1.0 "	2.3
		4.1 gel	8.7‖	3.0 gel	4.7			3.0 gel	8.8	2.0 gel	12.6	2.0 HOPG	0.0
										4.1 "	11.0	2.0 "	0.0
JF	27	1.1 OPG	0.0	5.6 OPG	0.0	1.0 gel	3.5	4.2 OPG	4.3	1.1 OPG	3.3	1.0 gel	3.5
		3.3 "	0.2			1.5 "	0.8	3.0 gel	8.8	3.4 OPG	2.2	1.0 "	2.2
		4.1 gel	4.2‖	3.0 gel	4.8					2.0 gel	11.5	2.0 HOPG	0.0
										4.1 "	11.5	2.0 "	0.0

* 3.0 ml. used for analyses except where noted.

‡ Only 1st absorption values reported here.

§ Results of a 3rd absorption with clinical OPG were negative.

‖ 1.5 ml. used for analysis.

In the repeat study when three different antigens were used for immunization, the preimmunization sera were analyzed as follows: Group 1—(clinical OPG); to 3.0 ml. aliquots 3.4 and 10.1 μg. OPG N were added. After incubation and centrifugation as described above, the supernatants were absorbed successively with clinical OPG and gelatin. Additional aliquots were absorbed initially with gelatin.

TABLE III

*Response of Human Volunteers to Injection of High Molecular Weight OPG S 230 X (Group 2)**

Subject	No.	Preimmunization absorption						3 wk. postimmunization absorption‡		7 wk. postimmunization absorption			
		1st		2nd		3rd				1st		2nd§	
		Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum
		μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg.
CB	31	1.1 HOPG	6.6	1.7 HOPG	7.2	1.0 gel	1.9	1.7 HOPG	6.5	1.7 HOPG	8.7	2.0 HOPG	2.4
		3.8 "	15.3	1.7 "	1.9	1.5 "	0.3	5.6 "	7.7	2.8 "	10.1	2.0 "	1.0
		4.1 gel	15.8‖	3.0 gel	4.6			2.8 gel	9.0	2.0 gel	10.3	1.0 gel	0.6
										4.1 "	7.2		
JF	32	1.1 HOPG	6.1	1.7 HOPG	3.6	1.0 gel	2.5	1.7 HOPG	9.3	1.7 HOPG	10.2	2.0 HOPG	3.0
		3.8 "	13.9	1.7 "	2.2	1.5 "	1.0	5.6 "	13.1	2.8 "	13.8	2.0 "	2.1
		4.1 gel	15.5‖	3.0 gel	5.3			2.8 gel	12.3	2.0 gel	13.5	1.0 gel	1.8
										4.1 "	10.6		
CT	33	1.1 HOPG	2.8	1.7 HOPG	2.4	1.0 gel	0.6	1.7 HOPG	5.4	1.7 HOPG	8.8	2.0 HOPG	2.9
		3.8 "	4.5	1.7 "	0.1	1.5 "	0.8	5.6 "	3.3	2.8 "	10.9	2.0 "	0.3
		4.1 gel	5.8‖	3.0 gel	2.0			2.8 gel	5.5	2.0 gel	11.2	1.0 gel	1.4
										4.1 "	9.3		
FK	34	1.1 HOPG	4.5	1.7 HOPG	4.4	1.0 gel	1.4	1.7 HOPG	10.9	1.7 HOPG	9.5	2.0 HOPG	1.9
		3.8 "	8.4	1.7 "	0.4	1.5 "	0.5	5.6 "	14.6	2.8 gel	12.6	2.0 "	2.8
		4.1 gel	8.8‖	3.0 gel	2.6			2.8 gel	13.4	2.0 "	12.1	1.0 gel	2.2
										4.1 "	11.7		
RSS	35	1.1 HOPG	4.9	1.7 HOPG	7.7	1.0 gel	1.8	1.7 HOPG	9.6				
		3.8 "	12.5	1.7 "	1.4	1.5 "	0.7	5.6 "	10.2				
		4.1 gel	13.3‖	3.0 gel	2.6			2.8 gel	11.0				
CLK	36	1.1 HOPG	4.4	1.7 HOPG	4.6	1.0 gel	1.4	1.7 HOPG	5.3				
		3.8 "	9.3	1.7 "	0.4	1.5 "	0.0	5.6 "	6.4				
		4.1 gel	9.9‖	3.0 gel	3.8			2.8 gel	8.5				
RB	37	1.1 HOPG	4.8	1.7 HOPG	4.6	1.0 gel	1.0	1.7 HOPG	2.9	1.7 HOPG	5.7	2.0 HOPG	1.1
		3.8 "	9.6	1.7 "	0.6	1.5 "	0.0	5.6 "	1.5	2.8 "	6.1	2.0 "	0.7
		4.1 gel	8.8‖	3.0 gel	4.8			2.8 gel	2.6	2.0 gel	8.5	1.0 gel	1.1
										4.1 "	5.0		

* 3.0 ml. used for analyses except where noted.

‡ Only 1st absorption values reported here.

§ Results of a third absorption with clinical OPG were negative.

‖ 1.5 ml. used for analysis.

Group 2 (high molecular weight OPG). 3.0 ml. aliquots were set up with 3.3 and 11.4 μg. N fractionated OPG. The supernatants were successively absorbed with five μg. fractionated OPG N and 3 to 4.5 μg. gelatin N. Here, too, additional aliquots were absorbed with gelatin.

Group 3 (gelatin). 3.0 ml aliquots were set up with 4.1 and 8.3 μg. gelatin N. After analysis of the precipitates, the supernatants were absorbed with 8.3 and 4.1 μg. gelatin N respec-

TABLE IV

*Response of Human Volunteers to Injection of Gelatin L 2160**

Subject	No.	Preimmunization absorption						3 wk. postimmunization absorption‡		7 wk. postimmunization adsorption			
		1st		2nd		3rd				1st		2nd§	
		Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum	Antigen N added per ml. serum	Antibody N precipitated per ml. serum
		μg.	μg.	μg.	μg.	μg.	μg.	μg.	μg	μg.	μg.	μg.	μg.
JD	41	1.4 gel	11.9	2.8 gel	3.2	4.2 OPG	0.0	3.1 gel	6.2	2.0 gel	10.0	1.0 gel	1.5
		2.8 "	13.1	1.4 "	0.9	3.3 HOPG	0.0	5.5 "	6.3	4.1 "	—		
		4.5 OPG	6.2‖					4.2 OPG	3.5	3.3 OPG	5.5	1.0 gel	0.0
										2.8 HOPG	9.6	1.0 "	0.0
HH	42	1.4 gel	6.7	2.8 gel	0.0	4.2 OPG	0.0	3.1 gel	6.9				
		2.8 "	6.8	1.4 "	0.0	3.3 HOPG	0.0	5.5 "	7.5				
		4.5 OPG	3.0‖					4.2 OPG	4.8				
HH	43	1.4 gel	9.7	2.8 gel	2.1	4.2 OPG	0.0	3.1 gel	7.9	2.0 gel	10.0	1.0 gel	3.1
		2.8 "	11.5	1.4 "	0.8	3.3 HOPG	0.0	5.5 "	8.5	4.1 "	12.3	1.0 "	3.2
		4.5 OPG	4.0‖					4.2 OPG	4.2	3.3 OPG	4.5	1.0 "	3.5
										2.8 HOPG	12.2	1.0 "	1.5
RD	44	1.4 gel	12.1	2.8 gel	2.4	4.2 OPG	0.0	3.1 gel	8.9	2.0 gel	13.1	1.0 gel	3.6
		2.8 "	13.1	1.4 "	1.2	3.3 HOPG	0.0	5.5 "	9.1	4.1 "	11.2	—	—
		4.5 OPG	5.5‖					4.2 OPG	5.8	3.3 OPG	6.1	1.0 gel	1.9
										2.8 HOPG	15.7	1.0 "	0.0
RC	45	1.4 gel	6.3	2.8 gel	0.4	4.2 OPG	0.0	3.1 gel	8.5	2.0 gel	8.8	1.0 gel	1.7
		2.8 "	6.9	1.4 "	0.0	3.3 HOPG	0.0	5.5 "	8.5	4.1 "	6.8	—	—
		4.5 OPG	2.9‖					4.2 "	4.6	3.3 OPG	3.8		
										2.8 HOPG	8.9		
EH	46	1.4 gel	10.4	2.8 gel	2.6	4.2 OPG	0.0	3.1 gel	3.1	2.0 gel	9.5	1.0 gel	2.4
		2.8 "	11.5	1.4 "	0.9	3.3 HOPG	0.0	5.5 "	3.0	4.1 "	7.4	—	—
		4.5 OPG	4.5‖					4.2 "	2.1	3.3 OPG	3.6	1.0 gel	3.5
										2.8 HOPG	9.6	1.0 "	0.4
RB	47	1.4 gel	12.7	2.8 gel	3.7	4.2 OPG	0.0	3.1 gel	3.9	2.0 gel	9.0	1.0 gel	0.9
		2.8 "	16.5	1.4 "	1.9	3.3 HOPG	0.0	5.5 "	4.7	4.1 "	7.3	1.0 "	0.2
		4.5 OPG	5.9‖					4.2 "	2.8	3.3 OPG	3.2	1.0 "	2.4
										2.8 HOPG	9.2	1.0 "	0.0

* 3.0 ml. used for analyses except where noted.

‡ Only 1st absorption values reported here.

§ Results of a 3rd absorption with clinical OPG were negative.

‖ 1.5 ml. used for analysis.

tively, followed by absorption with either clinical OPG or high molecular weight OPG. Once again this procedure was repeated initially using clinical OPG.

The data giving the amounts of antigen added to the original serum and supernatant for each analysis and the antibody N precipitated during each absorption for the pre-, and the 3, and 7 week postimmunization bleedings are given in Tables II to IV.

Quantitative precipitin curves (9) were set up with the sera obtained from those volunteers who had shown the best antibody responses. The sera were calibrated using all three antigens irrespective of the one used for immunization. Tables V and VI show the data with the sera from subject 19 and the pool in Group 1.

TABLE V

Antibody Nitrogen Precipitated by Gelatin and Its Derivatives from 3.0 ml. Human Antiserum (Anti-OPG) from Subject RRB, no. 19

OPG N added per ml. serum	Antibody N precipitated per ml. serum	HOPG N added per ml. serum	Antibody N precipitated per ml. serum	Gel N added per ml. serum	Antibody N precipitated per ml. serum
μg.	μg.	μg.	μg.	μg.	μg.
2.3	1.4	0.5	2.1	1.4	7.5
3.4	2.0	1.1	4.4	2.8	10.9
5.6	4.5	2.2	7.0	5.5	11.3
8.5	2.7	3.6	8.2	11.0	11.2
11.3	2.6	5.4	8.3	16.5	6.4
16.9	1.4	24.4	6.7	55.0	1.0

TABLE VI

Antibody Nitrogen Precipitated by Gelatin and Its Derivatives from Human Antiserum (Group 1)

OPG N added per ml. serum*	Antibody N precipitated per ml. serum	HOPG N added per ml. serum‡	Antibody N precipitated per ml. serum	Gel N added per ml. serum‡	Antibody N precipitated per ml. serum
μg.	μg.	μg.	μg.	μg.	μg.
0.7	1.0	0.9	1.4	0.7	3.6
1.9	4.5	1.9	6.7	2.0	14.0
2.8	6.0	3.5	10.9	4.0	16.5
4.2	7.7	5.6	15.5	5.5	16.0
7.0	10.2	8.4	15.1	8.3	15.4
11.3	11.1	13.4	10.4	13.7	14.2
16.9	10.4	28.0	7.5	27.5	12.5
28.2	7.5	54.2	0.0	41.3	12.0
33.7	4.3	67.6	0.0	69.3	9.6
56.4	0.6				

* 3.0 ml. serum used for each analysis.

‡ 1.5 ml. serum used for each analysis.

Hydroxyproline Analysis.—In order to measure the OPG and/or gelatin incorporated into the specific precipitates advantage was taken of the high concentration of hydroxyproline in gelatin and its derivatives. The method of Neuman and Logan (10) was employed. Additional precipitates, in triplicate, were analyzed along with standards of both gelatin and hydroxyproline[3] each time. This was necessitated by the fact that the hydroxyproline curves were not the same each day.[4]

[3] The hydroxyproline standards were made from the material supplied by Pfanstiehl Chemical Co., Waukegan, Illinois.

[4] Similar observations have been noted by others (11).

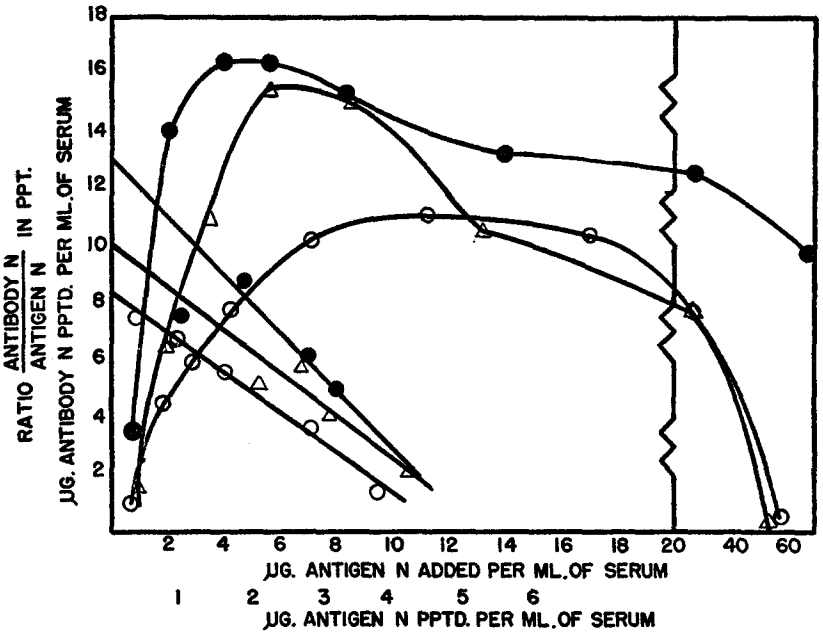

FIG. 2. Calibration curves of human antiserum (Group 1). O, clinical OPG; △, fractionated OPG; ●, pigskin gelatin.

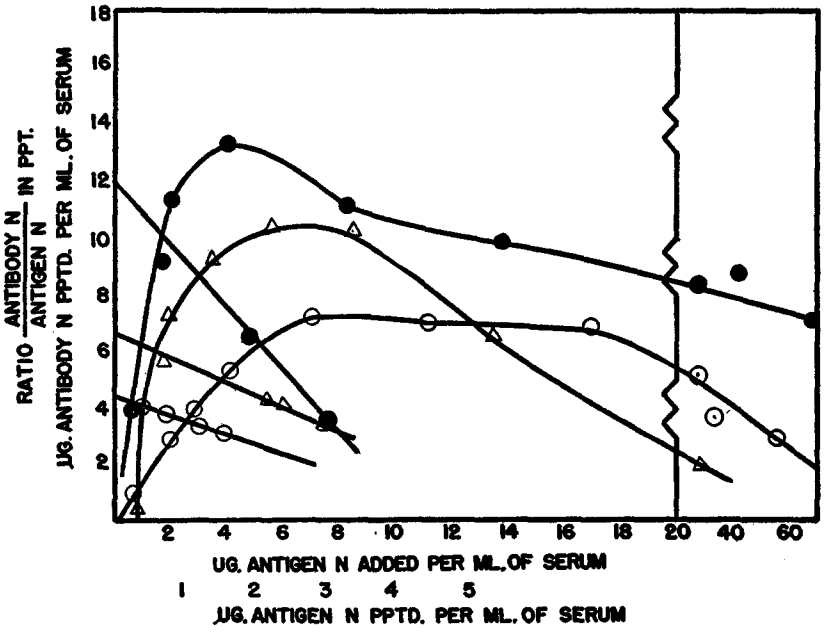

FIG. 3. Calibration curves of human antiserum (Group 2). O, clinical OPG; △, fractionated OPG; ●, pigskin gelatin.

Although hydroxyproline analyses were performed on many precipitates obtained with preimmunization sera the values presented in Table VII and Figs. 2 and 3 refer only to the postimmunization sera.

Solubility of Specific Precipitates.—In order to determine the solubility of gelatin–anti-gelatin precipitates in excess antigen additional precipitates were formed. They were washed as described above and then resuspended overnight at room temperature in either 1.0 ml. of gelatin, formalinized gelatin, modified fluid gelatin,[5] bovine serum albumin,[6] ovalbumin,[7] containing 1 mg. N/ml. or in 1 ml. of 0.9 per cent NaCl which served as the control. The precipitates were then centrifuged, washed with chilled saline, dissolved to 2.5 ml. and 2.0 ml.

TABLE VII

Analysis of Precipitates from Group 2 Serum for Presence of Antigen

Antigen N added per ml. serum	Antigen N precipitated from hydroxyproline data	Antigen precipitated	Antibody N precipitated (from precipitin curves)	Antibody N precipitated / Antigen N in precipitate
μg.	μg.	*per cent*	μg.	
OPG 1.1	0.4	36	1.5	4.1
2.8	0.7	25	2.7	3.8
5.6	1.2	22	4.1	3.4
7.1	1.5	21	4.8	3.2
HOPG 1.1	0.7	66	4.0	5.6
3.5	2.2	63	9.4	4.3
5.6	2.4	43	10.4	4.3
8.4	2.9	35	10.2	3.5
11.2	2.1	18	8.2	3.9
GEL 1.1	0.7	64	6.5	9.3
2.8	1.3	47	12.7	9.8
5.5	1.9	35	12.5	6.6
8.3	3.0	36	11.0	3.7

aliquots taken for analysis of the tyrosine color produced by the Folin-Ciocalteu method. The results were conclusive that the precipitates dissolved only in gelatin or formalin-treated gelatin.

Rabbit Anti-HGG.—Antisera against human gamma globulin were obtained by immunization of rabbits with either Cutter's[8] or Lederle's[9] immune serum globulin as described in previous publications (12).

Resuspension of Specific Precipitates in Anti-HGG Sera. One of the methods used to substantiate the fact that the protein being precipitated by gelatin and/or its derivative was indeed antibody (gamma globulin) was to resuspend precipitates in rabbit anti-HGG sera. Gelatin-antigelatin precipitates were formed by adding increasing amounts of gelatin to

[5] Kindly furnished by Dr. D. Tourtellotte of the Knox Gelatin Company, Camden, New Jersey.

[6] Armour recrystallized BSA Lot No. 128-75, The Armour Laboratories, Chicago.

[7] Prepared according to reference 25.

[8] Cutter Laboratories, Berkeley, California.

[9] Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

varying volumes of human antigelatin sera. The precipitates which formed were washed six times to eliminate the slightest possibility of any serum proteins remaining adsorbed to the precipitates. The precipitates were then resuspended in 1.0 ml. of the rabbit anti-HGG serum at 5°C. Additional precipitates were resuspended in 1.0 ml. of normal rabbit serum which served as the respective controls. Both the normal rabbit serum and rabbit anti-HGG were decomplemented to remove the possibility of complement N interfering with the results (13). After 8 days in the refrigerator, during which time the precipitates were resuspended twice daily, the tubes were centrifuged and washed as mentioned above. The precipitates were dissolved and analyzed for Folin color at 7500 A as described above. The results are given in Table VIII.

Passive Transfer (Anaphylaxis) Studies.—Eight ml. of human serum containing 5 μg. of antibody (antigelatin) N per ml. were injected into each of six guinea pigs weighing about 250 gm. (14). A "shocking" dose of 1 ml. of gelatin containing 1 mg. N was injected intravenously 48 hours later. As no anaphylaxis ensued the guinea pigs were injected with 60 μg. of rabbit antibody N against BSA. This was to see whether the guinea pigs were refractory The challenging dose of BSA was 1 mg.

TABLE VIII

Optical Density at 7500 A of Gelatin-Antigelatin (Human)

Resuspended in	
Normal rabbit serum	Rabbit Anti-HGG sera
0.028	0.040
0.141	0.190
0.164	0.250
0.205	0.300

RESULTS

Table I summarizes the results obtained in 20 individuals in the initial study involving clinical OPG. In all the data presented the exceedingly small contribution of Folin color given by gelatin or its derivatives has been neglected in calculating antibody values. Analyses of varying concentrations of gelatin and OPG were performed by the Folin-Ciocalteu method. As was to be expected, owing to the virtual absence of tyrosine in the proteins, insignificant optical densities were obtained.

Quantities of antibody nitrogen below 2 μg. per ml. are not considered significant. The data in the preimmunization sera show that 13 of the 20 volunteers (65 per cent) had significant amounts of protein precipitable by OPG. In 13 of the postimmunization sera the precipitable antibody had increased by at least 2 μg. N. In one instance (No. 2) there was a rise in antibody N from 2 to 12 μg. per ml. In 3 sera there was no significant change in antibody level whereas one subject actually showed a decrease in antibody level. Three of the postimmunization sera were lost. There were no marked skin reactions (wheal and erythema type) either before or after immunization. The concentration of

OPG injected (2 to 25 mg./ml) had no effect on the magnitude of the antibody response. It was somewhat surprising to find antibody present in the preimmunization sera. The possible sources of this antibody will be discussed later.

The sera of the 500 ml. bleedings obtained from the volunteers (Nos. 2, 6, 13, and 19) were calibrated with clinical OPG, the fractionated OPG and gelatin. Only the data obtained with No. 19 is presented as it was quite representative of the others. From Table V and Fig. 1 it is quite evident that we are dealing with a protein—antiprotein system. The shapes of these curves and those to be presented later are typical of a polydisperse and/or denatured antigen. (For complete discussion of this see reference 9). The precipitation of antibody from the serum by the different antigens increased with the molecular size of the antigen; *i.e.*, gelatin precipitated more antibody than did fractionated OPG which in turn precipitated more antibody than did clinical OPG. This will be reiterated in the results obtained in the repeat experiment.

Tables II, III, and IV present the results of the many analyses of the three different groups of volunteers used in the repeat experiment. Group 1 was injected with clinical OPG, Group 2 with the fractionated (high molecular weight) OPG, and Group 3, was injected with the gelatin from which the OPG was made. For the sake of clarity and to avoid generalization it is best to present the results of each group separately.

Group 1. The analyses of the preimmunization sera with OPG and gelatin indicate that gelatin precipitates much more antibody than does clinical OPG. Although the absorption of the supernatants from the OPG tubes with more OPG precipitates more antibody, still there is considerably less antibody precipitated than by the gelatin alone. In most cases small amounts of gelatin added to the second supernatant precipitated additional antibody. Of considerable interest is the fact that the total antibody N precipitated in two absorptions with OPG (1.1 μg. OPG N/ml. serum followed by 5.6 μg. OPG N/ml) is equal, within experimental error, to the amount precipitated by 3.3 μg. OPG N/ml. serum originally.

In most cases two absorptions with gelatin precipitate significantly more antibody than did the OPG plus small concentrations of gelatin. A third absorption of the sera analyzed with gelatin with OPG gave insignificant precipitates.

The results obtained on the 3 week postimmunization bleedings were somewhat surprising in that five out of the seven individuals had a lower antibody level than before injection of OPG. Further bleedings were therefore taken at the time the 3 week bleeding values were obtained. This, in all cases, was 7 weeks after the last injection. For brevity, only the results of two absorptions will be presented for the 7 week postimmunization bleedings. Of the five individuals who showed decreased antibody in the 3 week postimmunization bleedings four had significant increases in antibody N on the 7 week bleedings.

Group 2.—The analyses of the preimmunization sera with fractionated OPG

and gelatin indicate that gelatin precipitates only a few more μg. of antibody N from the various sera than does the fractionated OPG. Moreover here too the total antibody precipitated by the absorption of antibody first with 1.1 μg. N high molecular weight OPG followed by another precipitation with 1.7 μg. N fractionated OPG was equal to the amount of antibody N precipitated by a single precipitation with 3.8 μg. N fractionated OPG.

The results on the 3 week postimmunization bleedings in this group also indicated that of the seven volunteers injected with fractionated OPG only one showed a significant increase over the preimmunization antibody levels. Three showed a significant decrease and three did not change significantly in their antibody values.

Of the five volunteers from whom serum was obtained 7 weeks after the last injection two showed a significant increase and two showed a decrease in antibody levels. Of interest is subject 32 who showed no significant change in antibody level in any of the three bleedings.

Group 3.—The analyses of the preimmunization sera with gelatin and clinical OPG again showed that gelatin precipitates more antibody than does clinical OPG. The total antibody precipitated by the absorption of antibody first with 1.4 μg. gelatin N followed by another absorption with 2.8 μg. gelatin N was equal to the amount precipitated by 2.8 μg. gelatin N followed by 1.4 μg. gelatin N.

In only 1 individual (No. 45) in the 3 week postimmunization bleeding was there any increase in antibody. No. 42 showed no change and all the others had decreases in antibody level.

Of the six volunteers from whom serum was obtained at the 7 week bleeding three exhibited increases, two showed no change, and one still had antibody levels below the preimmunization values.

The 7 week bleedings in this group were analyzed with gelatin, fractionated OPG, and the clinical OPG. In most cases the antibody N precipitated by gelatin and the high molecular weight OPG were about the same whereas the clinical OPG precipitated ⅓ to ½ as much antibody as the other antigens.

It should be mentioned that in addition to the results on medical volunteers tabulated in this paper, similar results have been obtained in another 40 volunteers. The serum from the 500 ml. bleedings of the two best reactors in each group were pooled into their respective groups. Results of the calibration curves obtained with the pools of Groups 1 and and 2 are presented in Figs. 2 and 3. The data used to prepare Fig. 2 are presented in Table VI. From these data again it is fairly certain that one is dealing with a protein-antiprotein system in which the antigen is polydisperse and/or denatured. That gelatin precipitates more antibody than the fractionated OPG, which in turn precipitates more than the clinical OPG, is further substantiated by these data.

As the precipitates were exceedingly gelatinous in character there was no

doubt that the antigens were in the precipitates. Preliminary analyses of the precipitates from some of the preimmunization sera for hydroxyproline also indicated that gelatin was indeed in the precipitate. However, in order to get a quantitative estimation of the amount of the added antigen which was incorporated into the precipitate, additional precipitates were analyzed for hydroxyproline. The results presented in Table VII (Group 1) indicate that in antibody excess about 33 per cent of the OPG, 65 per cent of the fractionated OPG, and about 65 per cent of the gelatin added are incorporated into the precipitates. Similar results were obtained with the other two pools of human serum. With Group 2 serum 80 per cent of the gelatin was incorporated into the precipitates in antibody excess. As all these antigens are polydisperse one can assume that those molecules of highest molecular weight are present in the antigen-antibody complex. Similar results were recently obtained by Kabat *et al.* (15) using the dextran-antidextran system. When the clinical type and fractionated dextrans containing C^{14} were added to human antidextran sera in the region of antibody excess 60 to 100 per cent of the added radioactivity was precipitated.

From the amounts of antigen added to the serum the theoretical amount of antibody N which should be precipitated can be read off the calibration curves (9). This information allows a calculation of the antibody N/antigen N ratios. When the ratios of antibody N/antigen N in the precipitates were plotted against the antigen N in the precipitates fairly straight lines were obtained. The initial combining ratios (2R) of the different antigens with the Group 1 serum were 8.3 for OPG, 10.2 for the fractionated OPG, and 13 for the gelatin. With the group 2 serum the 2R values were 4.4, 6.7, and 11.7 respectively. That fairly straight lines were obtained would lend credence to the fact that one is dealing with a true antigen-antibody reaction.

The gelatin anti-gelatin precipitates were soluble only in excess gelatin, thus fulfilling a very important criterion of an antigen-antibody precipitate; *i.e.*, that it be soluble in excess antigen. The results obtained with the formalinized gelatin and modified fluid gelatin were in accord with unpublished experiments from this laboratory on the cross-reactivities of various gelatin derivatives with antigelatin sera (16). As a complete report will be submitted shortly it is only necessary to state here that formalinized gelatin cross-reacted extensively with antigelatin sera whereas modified fluid gelatin did not react.

That the protein precipitated with gelatin resembled human gamma globulin is suggested by the fact that the precipitates picked up additional protein material from the rabbit anti-HGG serum (Table VIII). Although offhand one might expect the gamma globulin in the gelatin-antigelatin precipitates would react with more antigamma globulin, it should be remembered that the gelatin may be masking some of the sites in the gamma globulin molecules which are capable of reacting with the antihuman gamma globulin molecules.

Additional proof that the gelatin-antigelatin precipitates contained gamma globulin was furnished by the ultraviolet absorption spectra of the dissolved precipitates which resembled closely that of human gamma globulin.

DISCUSSION

Before discussing any of the implications of these findings on the antigenicity of gelatin in man the obvious question arises as to why had it not been observed heretofore. Previous investigations of this problem did not utilize the micro-precipitin techniques used in this study. Waksman and Mason (2) detected some increase in the nitrogen adsorbed on collagen particles when added to human sera. However, this increase was well within the limits of this method of analysis for nitrogen. In addition, complement fixation tests which were used by previous investigators would be of little value as there were no significant differences in the antibody levels before or after decomplementation with an ovalbumin (Ea)–rabbit anti-Ea system (17). The recent paper of Watson, Rothbard, and Vanamee (18 *a*), which is a more intensive investigation of the work of Loiseleur and Urbain (18 *b*), indicates that rat collagen (acid-soluble form) is antigenic in rabbits. The method for estimation of the antibodies was complement fixation. It is quite possible that the collagen-anticollagen system fixes complement whereas the gelatin-antigelatin precipitate does not. From recent experiments in this laboratory there seem to be some immunochemical differences between gelatin and collagen. Another very likely possibility as to why no precipitation was observed before, is that in previously reported work the reaction was observed only for 48 hours. In most of the cases studied here precipitation of the antigen-antibody aggregates did not occur for 3 to 6 days. At this point it may be worth mentioning that the speed for precipitation was determined not only by the antibody level in the serum but also by the antigen added. Gelatin, in many cases caused precipitation overnight, whereas the clinical OPG set up at the same time did not precipitate for 4 to 5 days. With other sera the gelatin did not precipitate before 4 days.

At this point it might be questioned whether or not we are measuring antibodies to gelatin or to some impurity in the gelatin. Although the presence of hydroxyproline (gelatin) in the precipitates should be conclusive evidence, further evidence was obtained through the use of gelatins from several species (16). These various gelatins were almost equally effective in precipitating antibody from different sera. Complete data on these reactions will be presented in another communication. That all these gelatins would have the same impurity is fairly unlikely. However the possibility that gelatin or its derivative is cross-reacting with antibody to collagen or some degradation product of collagen cannot be overlooked. Experiments are underway to compare by these quantitative immunochemical techniques the "procollagens" (soluble collagen) and collagens from the various species with their respective gelatins.

The next question of importance is what is the source of the antibodies to gelatin in the pre- "immunization" sera. At least two possibilities exist. The individuals may produce antibody against the gelatin present in most diets or else the antibodies may be made to the naturally occurring collagen in the connective tissue. It is quite plausible that by the action of certain enzymes collagen might be converted into a gelatin-like substance which then can induce the formation of antibodies. In order to test these possible explanations it is hoped to analyze sera from the following type individuals: infants who have never been fed gelatin or meat products, true vegetarians, and individuals suffering from what has been termed "collagen" disease such as lupus erythematosis, disseminated lupus, rheumatoid arthritis, and rheumatic fever. Although similar studies were initiated by Waksman and Mason (2), their method of analysis was not sensitive enough to detect 20 μg. of antibody N.

The data presented here to substantiate the fact that there are antibodies to gelatin in human sera may seem a bit voluminous. However, it should be realized that one of the theories concerning the antigenicity of proteins has been based on the fact that gelatin could not be shown to be antigenic. The lack of tyrosine residues in the molecule (19), the denatured state in which gelatin exists (20), and the high concentration of glycine which does not allow the molecule to attain a rigid backbone structure (21) have been said to account for the non-antigenicity of the gelatin molecule. By an entirely different method, namely the hemagglutination technique (22) which can measure as little as 0.006 μg. antibody per ml. serum, Grabar has recently confirmed the findings presented here concerning the presence of significant amounts of antibodies to gelatin in normal sera (23). In view of the data presented here and the confirmation by Grabar, some of the criteria for antigenicity of a protein molecule will have to be revised.

Since gelatin or its derivatives have been proposed as plasma expanders, the question arises as to the advisability of employing a plasma substitute which is antigenic. Under proper medical supervision two good reactors to gelatin were given reinjections of gelatin. No untoward reactions were observed at all.

It is worth recalling here an idea proposed by Grabar that some antibody molecules may not necessarily be functioning as part of a protective mechanism but rather as a transporting system involved in carrying materials to sites for further reaction (24). That the antibodies to gelatin are instrumental in the metabolism of gelatin and/or collagen is quite plausible.

The findings presented here and in the next paper which show that many normal sera have antibodies to gelatin make available immunochemical techniques for studying problems connected with the structure of the gelatins, soluble collagens (procollagens), and insoluble collagens from many species. Applications of these techniques to a study of "collagen" disease can also be visualized. Investigations along these lines are contemplated.

SUMMARY

It has been demonstrated that there are normally occurring antibodies to gelatin in human sera. Immunization with gelatin can in many cases increase the antibody level. The presence of these antibodies does not result in cutaneous reactions of the wheal and erythema type after injection of antigen.

Many of the properties of the gelatin-antigelatin reaction and the precipitates formed are those of a truly specific antigen-antibody aggregate.

Explanations have been advanced both as to the possible sources of the gelatin antibody and its significance.

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