EQUILIBRIA IN PRECIPITIN REACTIONS.

The Coexistence of a Single Free Antigen and Its Antibody in the Same Serum.*

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The vagueness of the terms of serology connotes the difficulties with which the describers of this new knowledge have had to contend. Unable to use simple and definite chemical substances, and without the guidance of any general theory, they have had to employ mass adjectives to describe the reactions of a serum as a whole, and have gathered great numbers of observations without any plan for their coordination. Whenever, therefore, it is possible to repeat former experiments by substituting known factors in place of unknown mixtures, the work should be undertaken. Quantitative data will be obtained through these studies which will eventually allow serology to find a place in either chemistry or physics, or what seems almost self-evident, in physical chemistry. The colloidal state of matter has afforded so many analogies to the reactions of serology that the terms of colloid chemistry have already been applied with uncritical facility by immunologists to reactions which have not yet been studied with quantitative accuracy sufficient to indicate their true nature. The systematic attempt, however, to prove or disprove these colloidal analogies in serology will undoubtedly result in a unifying conception of the principles of the reactions of immunity.

The precipitation of protein by specific sera has many analogies with colloidal reactions and this phenomenon lends itself readily to investigation simplified by the use of at least a single pure substance.

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Zinsser[1] has emphasized the colloidal phases of the precipitin reaction, and recently Weil[2] has repeated the experiments, using crystallized egg albumin as the antigen. One of the phases of the precipitin reaction which has engaged particular attention is that in which the precipitinogen and the precipitin occur simultaneously but ununited in the same serum. This is easily demonstrable when whole serum or a mixture of proteins is used as the antigen. Weil, however, using crystalline egg albumin as the antigen was unable to demonstrate the coexistence of free antigen and antibody in the same serum. It is of considerable theoretical importance to confirm this finding. The results obtained by Weil are so little in accord with expectations based upon chemical or colloidal theories that it was considered advisable to repeat the experiments on precipitin reactions with crystalline egg albumin. The following studies, therefore, were carried out under the direction of Dr. Zinsser, with some modifications of recent work on this reaction.

HISTORICAL.

In 1902 Linossier and Lemoine,[3] Ascoli,[4] and Eisenberg[5] noticed that when foreign serum had been injected into rabbits in large doses the sera of these animals contained both antigen and antibody. They used whole sera as antigens. To demonstrate the simultaneous presence of free precipitable substance and its precipitant in the same serum, fractions of the serum were tested, first for antigen, by adding another antiserum, and second for precipitin by adding an homologous antigen. Gay and Rusk[6] have described the same phenomena, and by showing that the sera do not fix complement, although they contain antigen and antibody, they have furnished additional evidence of the ununited state of the substances.

Zinsser and Young[7] have pointed out that the association of free antigen and its antibody in serum, as shown by the failure of this serum to fix complement, is evidence that the reaction does not take place according to the law of mass

[1] Zinsser, H., Infection and Resistance, London and New York, 1914, 266.

[2] Weil, R., *J. Immunol.*, 1916, i, 19.

[3] Linossier, G., and Lemoine, G.-H., *Compt. rend. Soc. biol.*, 1902, liv, 85.

[4] Ascoli, M., *Münch. med. Woch.*, 1902, xlix, 1409.

[5] Eisenberg, P., *Centr. Bakteriol., 1te Abt., Orig.*, 1903, xxxi, 773.

[6] Gay, F. P., and Rusk, G. Y., *Univ. California Publications, Pathology*, 1912, ii, 59, 73.

[7] Zinsser, H., and Young, S. W., *J. Exp. Med.*, 1913, xvii, 396.

action. According to the formula of that law, this serum should not only contain free antigen and antibody, but also a certain quantity of the united complex. To explain the inhibition of the union of antigen and antibody in such sera Zinsser and Young[7] have drawn a strict parallel with the protective colloidal reactions. These experiments show that it is not improbable that the serum proteins act as protective colloids, preventing the precipitation of a protein by its antiserum, just as gum arabic will protect colloidal arsenic sulfide against the precipitating action of gelatin.

Von Dungern[8] confirmed the findings of Linossier and Lemoine but explained them according to a different hypothesis. He showed that whole serum consists of a number of protein complexes, all of which cause the formation of precipitin in the immunized animal. One portion of the protein mixture may have more antigenic properties than another, as a result of which partial precipitins of various strengths are produced for the whole serum. Von Dungern believed that these complex antigens and multiple antibodies reacted irregularly in such a way that while the supernatant fluid of a precipitin test would contain free antigens found in part in the original serum together with precipitins capable of flocculating the original serum, yet the antisubstances in the supernatant fluid were not strictly homologous and hence would not react with each other. His extensive experiments have had great influence upon subsequent opinions. He worked, however, entirely with multiple antigens and gave only inferences and analogies for the precipitin reactions with pure proteins.

At first glance it would seem as if the experiments in the precipitation of casein with lactoserum, reported by Müller,[9] were evidence that with purified antigens the phenomenon of Linossier and Lemoine did not occur. The supernatant fluids after his precipitin reactions never contained free antigen along with antibody. His experiments, however, are not relevant to the results of precipitin reaction with purified protein. Müller used whole milk as his casein, obviously a multiple antigen, and the degrees of dilutions used in the tests leave such large gaps that the zone in which antigen and antibody coexist ununited might have been overlooked.

Weil[2] confirms all the previous observations on the coexistence of free antigen and antibody in the supernatant fluids of precipitin tests when the whole serum, raw egg white, or any mixed protein is used. With purified egg albumin as antigen, the results were different. He states: "If this antigen is mixed in graded proportions with the serum of a rabbit immunized thereto, and the resulting precipitates are removed by centrifugation, the supernatant fluid never contains both antigen and antibody; either one is present alone." The protocols of Weil's precipitin tests allow him to draw sharp conclusions between the effect of 0.005 cc. and 0.004 cc. of the antigen, a delicacy of reaction which not all workers have

[8] von Dungern, F., *Centr. Bakteriol., 1te Abt., Orig.*, 1903, xxxiv, 355.

[9] Müller, P. T., *Centr. Bakteriol., 1te Abt., Orig.*, 1903, xxxiv, 48.

been able to attain. The crystalline egg albumin, prepared for him by Coca, was certainly as pure as it is possible to obtain this substance. It is not stated whether anaphylactic reactions were carried out with this particular preparation to determine whether it was thereby free from antigen complexes which cause reactions in animals sensitized with egg globulin. Weil,[2] however, referring to his earlier work with Coca[10] states that he "found that if egg albumin and egg globulin were separated by chemical means, and different rabbits were immunized to each substance, the resulting immune sera reacted powerfully each with its own antigen, and very weakly with the other antigen." This anaphylactic interrelation of egg proteins will be discussed later. It is significant here to note the apparent discrepancy between the results of these precipitin tests and anaphylactic reactions, although one antigen was used which admittedly gives some anaphylactic reaction with other proteins.

EXPERIMENTAL.

Sera capable of precipitating egg albumin were prepared by immunizing rabbits with intravenous injections of this protein. By varying the proportions of immune serum and solution of egg albumin in the precipitin tests, zones of dilution were obtained in which tests for free antigen and free antibody could be made. In addition, by testing the sera of immunized rabbits at certain intervals after the injection of albumin, the proportions and duration of circulating antigen and antibody could be studied.

The important material for these experiments is the antigen. Attempts to use casein failed because of the confusing precipitates caused by the calcium salts required in the tests with this protein. In some experiments, pure edestin from hemp-seed was used.[11] The results with this protein were definite. Because, however, of the slight solubility of edestin, work with it is somewhat difficult. Crystalline egg albumin was found to be most suitable for these studies. This substance was prepared[12] according to the method of Hopkins and Pinkus.[13] The whites of freshly laid eggs were carefully precipitated with ammonium sulfate and the albumin from these was

[10] Weil, R., and Coca, A. F., *Z. Immunitätsforsch., Orig.*, 1913, xvii, 141.

[11] The preparation was made by Dr. Harris of the Connecticut Agricultural Experiment Station.

[12] The substance was prepared under the direction of Mr. Balls of the Department of Physiological Chemistry of Columbia University.

[13] Hopkins, F. G., and Pinkus, S. N., *J. Physiol.*, 1898–99, xxiii, 130.

recrystallized three times. The solution of protein was dialyzed under toluene in the ice box to free it as much as possible from ammonium sulfate and acetic acid. The albumin was then dried *in vacuo* to a white glistening powder. The Zsigmondy "gold number"[14] for the preparation was 8, showing the usual protective value of pure egg albumin as a colloid. This preparation met the requirements for pure crystalline egg albumin and was probably as pure as it is possible to obtain a protein of this nature.

Anaphylactic tests were made in the hope of showing that this preparation of egg albumin was free from globulin or substances which had antigenic properties like globulin. Egg globulin was prepared from the first fraction of egg white precipitated by half saturation with ammonium sulfate. A solution of the precipitate was dissolved in distilled water and dialyzed against distilled water until the globulin began to flocculate. This material was centrifuged and the precipitate taken up in 10 per cent sodium chloride. This formed a turbid emulsion, free, however, from large particles. One series of guinea pigs weighing 250 gm. was sensitized by intravenous injections of varying amounts of crystalline egg albumin; another series by intravenous injections of egg globulin. After an interval of 26 days the animals were reinjected with protein (Table I).

The results of the experiments summarized in Table I show that preparations of egg albumin and egg globulin have a common antigenic factor, capable of producing mutual anaphylactic reactions. They confirm the statements of Wells[15] and Wells and Osborne,[16] who, after a long series of experiments concluded that: "In spite of the most careful separation of these two portions of egg white by means of ammonium sulfate precipitation, the resulting preparations each react almost as well against the other as against itself." Weil and Coca[10] report a somewhat similar experience with crystalline egg albumin when injected into animals sensitized to egg globulin. These reactions have been considered in detail in order to determine whether or not the anaphylactic test is a valid criterion of the purity of a pro-

[14] Schulz, F. N., and Zsigmondy, R., *Beitr. chem. Phys. u. Path.*, 1903, iii, 137.
[15] Wells, H. G., *J. Infect. Dis.*, 1911, ix, 147.
[16] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1913, xii, 341.

TABLE I.

*Anaphylactic Reactions with Solutions of Ovalbumin and Ovoglobulin from the Same Eggs. Guinea Pigs, Weight 200 to 250 Gm., Injected Intravenously on July 13, 1916. Second Injection on August 7, after an Interval of 26 Days.**

Guinea pig No.	Sensitizing dose.	Interval.	Second injection.	Result.
	Animals sensitized to crystalline ovalbumin.			
		days		
1	0.002 gm. of albumin.	26	0.1 gm. of albumin.	Convulsions. Died.
2	0.002 " " "	26	0.2 " " "	" " in 2 hrs.
3	0.001 " " "	26	0.1 " " "	Typical anaphylaxis. Died in 3 min.
4	0.001 " " "	26	0.1 " " "	Convulsions. Died in 3 min.
5	0.0005 " " "	26	0.1 " " "	Violent convulsions. Died in 2 min.
6	0.002 gm. of albumin.	26	0.1 gm. of globulin.	Convulsions. Died in 3 min.
7	0.002 " " "	26	0.1 " " "	Died in 30 min.
8	0.001 " " "	26	0.002 " " "	Diarrhea. Survived.
9	0.001 " " "	26	0.002 " " "	Sneezing, coughing. Survived.
	Animals sensitized to ovoglobulin.			
10	0.004 gm. of globulin.	26	0.1 gm. of globulin.	Convulsions. Died in 1 min.
11	0.002 " " "	26	0.002 " " "	" " " "
12	0.002 " " "	26	0.002 " " "	" Paralysis in hind legs. Died in 2 min.
13	0.001 " " "	26	0.002 " " "	Cough. Survived.
14	0.001 " " "	26	0.002 " " "	Convulsions. Died in 2 min.
15	0.0005 " " "	26	0.002 " " "	" " " "
16	0.002 gm. of globulin.	26	0.1 gm. of albumin.	Cough. Survived.
17	0.002 " " "	26	0.1 " " "	Sneezing, dyspnea. Survived.
18	0.001 " " "	26	0.1 " " "	Sprawling, twitching. Survived.
19	0.001 " " "	26	0.1 " " "	Cough. Survived.
20	0.0005 " " "	26	0.1 " " "	" "

* Separate syringes and needles were used for each protein.

tein, and in particular whether the anaphylactic interreactions of egg globulin and egg albumin exclude the latter from use as a single pure antigen. Crystalline egg albumin is acknowledged to be a pure protein.[15,17] Egg globulin, however, probably consists of ovomucin and ovalbumin admixed in indefinite proportions.[15] The impurity of the globulin renders the anaphylactic reactions without significance. In the protocols of Table I, it is seen that the albumin-globulin reactions are much weaker than those caused by the injection of the specific protein into a sensitized animal. This undoubt-

EXPERIMENT I.

Edestin as Antigen.

This experiment was done to show coexistence of edestin and its precipitin in the serum of an immunized rabbit.

Apr. 15–18, 1916. Serum from Rabbit 21, immunized to edestin, obtained 1 hour after an intravenous injection of 12 cc. of 0.027 per cent solution of edestin. This serum was kept for 3 days in the ice box before being tested for its content of antigen and antibody. Serum from Rabbit 22 also immunized to edestin, having a precipitin titer of 1:4,000. These sera were mixed in various proportions. The diluent was a mixture containing 0.9 per cent sodium chloride and 0.105 per cent sodium carbonate in order to keep the edestin in solution.

Tube.	Serum 21.		Edestin.	Chloride-carbonate solution.	Serum 22.		Precipitate.		
	Dilution.	Amount.			Dilution.	Amount.	Ring.	After ½ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	*cc.*	*cc.*		*cc.*			
For precipitin.									
1	Undiluted.	0.2	0.2	0.5			±	+	+
2	1 : 5	0.2	0.2	0.5			o	++	++
For antigen.									
3	Undiluted.	0.2		0.5	Undiluted	0.2	+	++	++
4	1 : 100	0.2		0.5	"	0.2	++	+	+
5	1 : 1,000	0.2		0.5	"	0.2	±	±	±
Controls.									
6	Undiluted.			0.7			o	o	o
7				0.7	"	0.2	o	o	o
8			0.2	0.7			o	o	o
9			0.2	0.5	1:4,000	0.2	±	+	+

[17] Schryver, S. B., General Characters of the Proteins, New York, 1909, 20.

edly indicates that the globulin preparation contained ovalbumin. Belief in the individuality of the crystalline egg albumin as an antigen rests upon its chemical characteristics.

Accepting, therefore, purified edestin and ovalbumin as single antigens, the experiments were conducted upon their specific precipitin reactions.

EXPERIMENT II.

Edestin as Antigen.

Apr. 20, 1916. Various quantities of edestin were added to its antiserum to find the zone of dilution *in vitro* in which antigen and antibody occur in supernatant fluids after the primary precipitin reaction.

Serum from Rabbit 22, immunized to edestin, having a titer of 1:2,000.

0.02 per cent solution of edestin mixed in various proportions with undiluted Serum 22.

Tube.	Serum 22, undiluted.	0.02 per cent solution of edestin.	Chloride-carbonate mixture.	Precipitate after 24 hrs. in ice box.	Supernatant fluids.	
					For antigen.	For precipitin.
					0.2 cc. of supernatant fluid plus 0.3 cc. of Serum 22.	0.2 cc. of supernatant fluid plus 0.3 cc. of edestin solution.
	cc.	*cc.*	*cc.*			
1	0.2	2		++	++++	o
2	0.2	1		++	++	±
3	0.2	0.5	0.3	+++	++	±
4	0.2	0.2	0.6	+++	+	+
5	0.2	0.1	0.7	++	Tr.	+
6	0.2	0.01	0.8	+	?	+
7	0.2	0.001	0.8	+	o	++
8	0.2	0.0001	0.8	±	o	++
9	0.2	0.00001	0.8	o	o	++
Controls.						
10	0.2		0.3	o	o	++
11		0.2	0.3	o	++	o

This experiment demonstrates the phase in the precipitin reaction in which a single antigen occurs simultaneously but ununited with its homologous antibody. In the table, the shaded columns overlap each other through the zone in which edestin and antiedestin were found free in the same fluid.

EXPERIMENT III.

Crystalline Egg Albumin as Antigen.

June 15–16, 1916. Various quantities of crystalline ovalbumin were added to its antiserum to find the zone of dilution *in vitro* in which antigen and antibody occur in the supernatant fluids after the primary precipitin reaction. Serum from Rabbit 23 immunized to crystalline egg albumin, having a titer of 1:10,000. 5 per cent crystalline egg albumin in normal salt solution was added in diminishing amounts to undiluted Serum 23 according to the following table.

Tube.	Serum 23, undiluted.	5 per cent crystalline egg albumin.	Normal salt solution.	Precipitate after 18 hrs. in ice box.	Supernatant fluids.	
					For antigen.	For precipitin.
					0.2 cc. of supernatant fluid plus 0.2 cc. of Serum 23.	0.2 cc. of supernatant fluid plus 0.2 cc. of crystalline albumin, diluted 1:10.
	cc.	*cc.*	*cc.*			
1	0.5	0.05	0.25	++	+++	+
2	0.5	0.01	0.25	+++	++	+
3	0.5	0.005	0.25	+++	++	++
4	0.5	0.001	0.25	++++	+	++
5	0.5	0.00075	0.25	+++	±	++
6	0.5	0.0005	0.25	++	o	++
7	0.5	0.0001	0.25	+	o	+++
8	0.5	0.00005	0.25	±	o	+++
Controls.						
9	0.5		0.25	o	o	++++
10		0.5	0.25	o	+	o
11		0.5	0.25	o	+	o

The overlapping shaded areas in the above table show the zone in the supernatant fluids in which free antigen and antibody (crystalline egg albumin and its precipitin) definitely coexist.

EXPERIMENT IV.

Crystalline Egg Albumin as Antigen.

This experiment was done to show coexistence of antigen and antibody in the circulation of an immune animal.

June 2, 1916. Serum from Rabbit 23, immunized to crystalline egg albumin, having a titer of 1:5,000 before the last injection of antigen. 10.45 a.m. 3 cc. of 5 per cent solution of crystalline egg albumin were injected intravenously into Rabbit 23. 11.30 a.m. Bled from carotid 15 cc. 3 p.m. Tests were carried out as follows.

Tube.	Serum 23A before injection.	Serum 23B after injection.	5 per cent solution of crystalline egg albumin.		Normal salt solution.	Normal rabbit serum.	Precipitate after ½ hr. at 37°C.
			Dilution.	Amount.			
	cc.	*cc.*		*cc.*	*cc.*	*cc.*	
For antigen in Serum 23 B.							
1	0.2	0.2					++++
For precipitin in Serum 23 B.							
2		0.2	Undiluted.	0.2			+++
3		0.2	1 : 10	0.2			++
4		0.2	1 : 100	0.2			o
Controls.							
5					0.2		o
6						0.2	o
7	0.2	0.2			0.2		o
8	0.2	0.2				0.2	o
9			Undiluted.	0.2	0.2		o
10			"	0.2		0.2	o
11					0.2	0.2	o

Tube 1 shows that the serum of Rabbit 23 contained antigen ununited with its antibody 45 minutes after an injection of crystalline egg albumin. Tubes 2, 3, and 4 show that during this time free precipitin was still present in the blood of the immune animal.

In several experiments conducted like this one, similar results were obtained, showing that the sera of rabbits immunized to egg albumin contain uncombined antigen and antibody in the circulation for approximately 48 hours after the last injection of the antigen.

In many of the precipitin reactions with crystalline egg albumin the maximum of precipitation occurred only when the albumin solution was diluted from 500 to 1,000 times. This phase of the precipitin reaction is commonly known as the prozone, and is most readily explained as a phenomenon of colloidal relationship. In this particular case it seemed desirable to investigate the inhibition of precipitation effected by the albumin preparation, to relate it, if possible, to the protective influence exerted by this emulsoid on a sensitive gold sol. To show this effect, Experiment V was performed.

EXPERIMENT V.

Protective Action of Egg Albumin.

June 1, 1916. This experiment was done to show the protective action of a solution of crystalline egg albumin upon the precipitation of human serum by its specific antiserum. To the serum from Rabbit 24, having a titer of 1:10,000 against human serum, various quantities of a 5 per cent solution of crystalline egg albumin were mixed with human serum. After ½ hour, anti-human serum from Rabbit 24 was added to these mixtures, as follows. The gold number of this preparation of egg albumin was 8.

Tube.	Human serum.		Anti-human Serum 24.		5 per cent solution of crystalline egg albumin.	Normal salt solution.	Precipitate after ½ hr. at 37°C.
	Dilution.	Amount.	Dilution.	Amount.			
		cc.		*cc.*	*cc.*	*cc.*	
1	Undiluted.	0.2	Undiluted.	0.2		0.2	++++
2	"	0.2	1:5	0.2		0.2	+++
3	1:100	0.2	1:5	0.2		0.2	++
4	1:1,000	0.2	1:5	0.2		0.2	+
5	1:5,000	0.2	1:5	0.2		0.2	+
6	1:10,000	0.2	1:5	0.2		0.2	o
7	Undiluted.	0.2		0.2	0.2		++++
8	"	0.2	1:5	0.2	0.2		++
9	1:100	0.2	1:5	0.2	0.2		++
10	1:100	0.1	1:5	0.1	0.2	0.2	±
11	1:100	0.1	1:5	0.1	0.4		o
Controls.							
12	Undiluted.	0.2				0.2	o
13	"	0.2			0.2		o
14				0.2	0.2		o
15				0.2		0.2	o

These tests (Tubes 7 to 11) show that the presence of sufficient egg albumin prevents the flocculation of human serum by its antiserum. This effect of the solution of egg albumin is in accord with its well known protective action on other colloids, particularly its protection of a sensitive gold sol. against precipitation by electrolytes. The protective action in the above tests is obviously stronger than that exerted by the sera and may explain the long prozones commonly found in precipitin reactions with solutions of crystalline egg albumin.

The hypothesis of von Dungern[8] is insufficient to explain the fact that a serum containing both antigen and antibody, though clear at first, undergoes spontaneous precipitation upon long standing.[1] As

this precipitation progresses the amount of antigen and antibody gradually diminishes. This phenomenon is a matter of common observation when the sera of animals immunized to a mixture of protein are studied at successive intervals after an injection of the proteins used as antigen. Since it was shown by Experiments I and IV that a simple antigen such as edestin or crystalline egg albumin remains ununited with its homologous precipitin in the circulation of an immune rabbit for at least 24 hours after the last injection of the protein, a serum was readily obtained in which the slow spontaneous union of a single antigen with its antibody could be observed. This series of tests was carried out as follows.

EXPERIMENT VI.

Spontaneous Precipitation in a Serum Containing Antigen and Antibody (Crystalline Egg Albumin and Its Precipitin).

July 27, 1916. Rabbit 25 immunized to crystalline egg albumin, having a serum with a titer of 1:25,000, was injected intravenously with 3 cc. of a 5 per cent solution of crystalline egg albumin. 1 hour later blood was drawn aseptically into sterile tubes. Serum obtained from this was divided into several lots and stored in sterile tubes in the ice box. Titrations for antigen and antibody were made as follows.

Tube.	Serum 25 after injection.	Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Normal salt solution.	Normal rabbit serum.	Precipitate.	
			Dilution.	Amount.			After ½ hr. at 37°C.	After 24 hrs. in ice box.
	cc.	*cc.*		*cc.*	*cc.*	*cc.*		
For antigen.								
1	0.2	0.2					+++	++++
For precipitin.								
2	0.2		1 : 10	0.2			++	++
3	0.2		1 : 100	0.2			+	+
4	0.2		1 : 500	0.2			+	+
5	0.2		1 : 1,000	0.2			+	+
6	0.2		1 : 5,000	0.2			+	+
7	0.2		1 : 10,000	0.2			o	±
Controls.								
8	0.2				0.2		o	Slight precipitate (spontaneous).
9			1 : 10	0.2		0.2	o	o
10			1 : 10	0.2	0.2		o	o
11	0.2					0.2	o	o
12		0.2				0.2	o	o
13		0.2	1 : 25,000	0.2			+	+

Tube 1 shows that the serum contained a considerable amount of antigen, while Tubes 6 and 7 show that the precipitin titer of the same serum equalled 1:10,000. Tubes 8 to 13 were used to show that no non-specific precipitation occurred in any of the components of the primary reaction. With the subsequent tests, this series of controls was repeated invariably. Their results were always as anticipated and they will be omitted in detail from the following protocols.

The batches of Serum 25, kept in the ice box, showed definite spontaneous precipitation at the end of 24 hours.

July 28. Titration of the antigen and antibody content of this serum was carried out as follows.

Tube.	Serum 25 after injection.		Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Precipitate.	
	Dilution.	Amount.		Dilution.	Amount.	After ½ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	*cc.*		*cc.*		
For antigen.							
1	Undiluted.	0.1	0.1			++	++++
2	1 : 10	0.1	0.1			+	+++
3	1 : 100	0.1	0.1			+	++
4	1 : 250	0.1	0.1			+	+
5	1 : 500	0.1	0.1			o	±
6	1 : 1,000	0.1	0.1			o	o
For precipitin.							
7	Undiluted.	0.1		1 : 1,000	0.1	+	+
8	"	0.1		1 : 5,000	0.1	±	±
9	"	0.1		1 : 10,000	0.1	o	o

This series of tests shows that the quantity of antigen present in Serum 25 (after injection) was sufficient to give a visible reaction when diluted 500 times. At the same time the precipitin titer of this serum containing free antigen was 1 : 1,000. In the previous 24 hours, however, while spontaneous precipitation had occurred in the serum, the precipitin titer decreased from 1 : 5,000 to 1 : 1,000.

Spontaneous precipitation continued, and at the end of 48 hours the following proportions of egg albumin and its precipitin were found to be present.

July 29. Serum 25 titrated as follows:

Tube.	Serum 25 after injection.		Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Precipitate.	
	Dilution.	Amount.		Dilution.	Amount.	After ½ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	*cc.*		*cc.*		
For antigen.							
1	Undiluted.	0.1	0.1			++	+++
2	1 : 10	0.1	0.1			+	++
3	1 : 100	0.1	0.1			±	+
4	1 : 250	0.1	0.1			o	+
5	1 : 500	0.1	0.1			o	o
For precipitin.							
6	Undiluted.	0.1		1 : 500	0.1	+	+
7	"	0.1		1 : 1,000	0.1	o	o
8	"	0.1		1 : 5,000	0.1	o	o

July 31. 96 hours after the serum had been obtained, the tubes were turbid with considerable flocculated material in the sediment. The turbidity disappeared on warming the serum, but the sediment remained, giving evidence of the specific nature of this spontaneous precipitate. Titration of the serum at the end of 96 hours gave the following values for its content of free antigen and antibody.

Tube.	Serum 25 after injection.		Serum 25 before injection.	5 per cent solution of crystalline egg albumin.		Precipitate.	
	Dilution.	Amount.		Dilution.	Amount.	After ½ hr. at 37°C.	After 24 hrs. in ice box.
		cc.	*cc.*		*cc.*		
For antigen.							
1	Undiluted.	0.1	0.1			+++	+++
2	1 : 10	0.1	0.1			++	++
3	1 : 150	0.1	0.1			+	+
4	1 : 200	0.1	0.1			+	+
5	1 : 300	0.1	0.1			o	o
For precipitin.							
6	Undiluted.	0.1		1 : 100	0.1	±	+
7	"	0.1		1 : 350	0.1	o	o

This shows that during the course of spontaneous precipitation in the serum, both egg albumin and its precipitin became decreased. At the end of 96 hours the antigen titer had fallen to 1 : 200 and the precipitin titer to 1 : 100.

Subsequent titrations were carried out to follow the decrease of these antisubstances coincident with progress of spontaneous precipitation in their serum.

At the end of 144 hours the serum no longer contained precipitin, while the antigen titer had decreased to 1 : 100.

At the end of 196 hours the precipitin was still zero, while the antigen titer remained constant at 1 : 100.

The results of these titrations of the supernatant serum during spontaneous precipitation are summarized in Table II, and represented graphically in the curve of the reaction (Text-fig. 1).

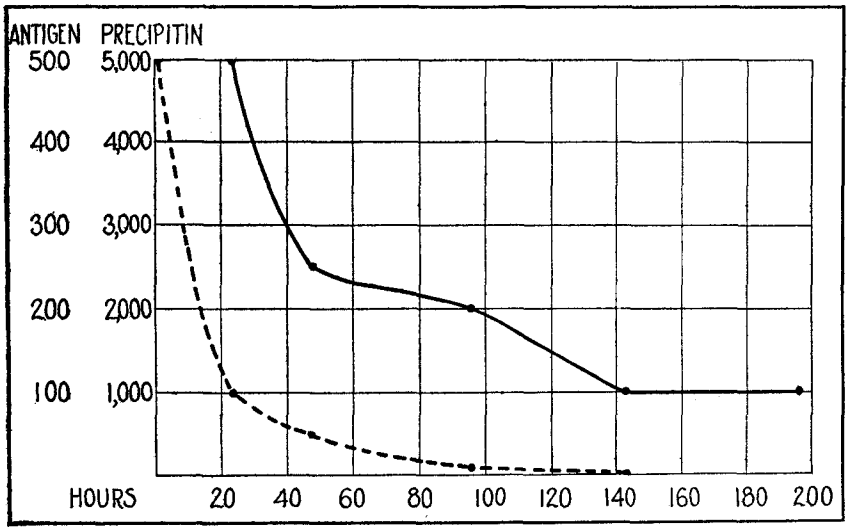
TABLE II.

Summary of Experiment VI.

Hours.	Precipitin.	Antigen.
2	1 : 5,000	?
24	1 : 1,000	1 : 500
48	1 : 500	1 : 250
96	1 : 100	1 : 200
144	0	1 : 100
196	0	1 : 100

The chart of the observed amounts of antigen and antibody remaining dissociated in the supernatant fluid of the precipitin reaction suggests at once that the process has taken place according to a definite law. As the amounts of antigen and precipitin are stated only in terms of the dilution of mixtures whose protein content was not accurately estimated it is not possible to analyze this curve in detail. Experiments are being undertaken, therefore, to determine quantitatively the mutual relationship of the factors of this reaction. While, however, calculations are not possible which would reveal the nature of this reaction, it is permissible to point out that the curve is not unlike that of some colloidal precipitations. In this case, the amount of precipitate together with the amount of precipitin remaining in solution in the presence of the precipitable substance is apparently a function of the concentration of the precipitable substance; *e.g.*, crystalline egg albumin, the colloidal properties of which are indisputable. Although the experiment was not conducted upon a quantitative basis, the regularity of the curve of its results is striking. From this feature it is a fair assumption to suppose that the reacting

antibodies were strictly homologous, and that the absence of irregularity in the curve is evidence that the antigen was a relatively simple substance. This confirms the chemical evidence of the purity of the preparation of crystalline egg albumin used as antigen.



—— Antigen (egg albumin).
- - - - - Precipitin.

TEXT-FIG. 1. Graphic representation of the results of Experiment VI, showing the changes in the quantities of antigen and antibody during spontaneous precipitation in a serum containing these substances. The quantities are expressed by titration values.

SUMMARY.

1. In these studies several phases of the precipitin reactions were investigated by the use of purified proteins as antigens. These preparations were edestin from hemp-seed and crystalline ovalbumin from fresh eggs. The ovalbumin, isolated by the method of Hopkins and Pinkus, was apparently as pure as is obtainable by chemical means. This albumin, however, produced moderately severe anaphylactic reactions in animals sensitized with ovoglobulin. Anaphylactic tests of the individuality of a protein cannot be any longer regarded as the criterion of the purity of the substance as an antigen.

Wells and Osborne[16] have shown that proteins of considerable chemical difference may have a common antigenic group which causes mutual anaphylactic reactions in animals sensitized to these proteins. In particular, as egg globulin is a mixture of proteins, one of which is undoubtedly egg albumin, anaphylaxis produced by injections of albumin into animals sensitized to the so called globulin offers no evidence for or against the purity of the albumin. The character of the curves shown in Text-fig. 1 confirms the assumption, based upon chemical data, that crystalline egg albumin is a single protein.

2. With edestin and crystalline egg albumin as antigens, phases in the precipitin reaction were found in which these substances and their specific precipitins could be demonstrated to be coexistent but ununited in the same serum.

3. When edestin or crystalline egg albumin is injected into a rabbit immunized thereto, the antigen may be found in the circulating blood during 48 hours after its injection, while at the same time the animal maintains a high titer of free precipitin in its blood.

4. When the pure protein antigen is mixed in proper proportions with the serum of a specifically immunized rabbit and the resulting precipitate removed by centrifugation, the supernatant fluid contains both antigen and antibody.

5. The serum drawn from a rabbit during the period in which free antigen and antibody are coexistent in the circulation undergoes slow spontaneous precipitation when kept in sterile tubes in the ice box. The curve of this reaction is reproduced as Text-fig. 1. The relationships of the parabola indicate that the interaction of antigen and antibody takes place according to a definite law. When sufficient quantitative data are obtained to allow an analysis of this curve, the formulas for this reaction will undoubtedly throw light upon the chemical or physical nature of the process.

6. The protective action of the solution of egg albumin as a third colloid preventing precipitation in a reaction between human serum and its antibody was readily demonstrated. This observation and the constancy of the long prozone in precipitin test with egg albumin are in accord with the protective action of ovalbumin upon colloidal gold.