THE ANTIGENIC PROPERTIES OF GLOBIN CASEINATE.*

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In previous articles we have considered the antigenic properties of the split products of casein (1), of a synthetic paranuclein (2), and of protamin caseinate (3). The main purpose of these studies has been to throw further light on the nature of biological specificity. As we have previously expressed ourselves, "One of the most promising methods of attack . . . would seem to lie in the search for newly formed antigenic properties in proteins compounded either of non-antigenic proteins, or of an antigenic with a non-antigenic protein." Our experiments in this direction hitherto have for the most part led to negative results. A combination of the amino acids of casein, in themselves non-antigenic, in the proportion in which they occur in casein, failed to produce an antibody. A compound of protamin (salmin), which is non-antigenic, with casein gave rise to an antibody for casein only. It is true that in our comparative studies of split paranuclein with synthesized paranuclein (2) we were apparently able to prove the genesis of antigenic properties in a protein (paranuclein) synthesized from non-antigenic proteoses (products of peptic digestion of casein) by the reversible action of pepsin.

Our experiments have incidentally thrown additional light on the relation that exists between the toxicity or non-toxicity of a protein and its antigenic properties, a relation that has been of particular importance in explaining anaphylaxis. In a general way it seems to be true from our own studies and from those of other investigators that split products of a non-toxic protein, hydrolyzed to

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the point of becoming toxic, lose their antigenic properties. This is not a matter, however, that we are prepared to discuss at the present time.

The present study deals with the antigenic properties of our second compound of casein with a non-antigenic protein. Robertson (4) has described the preparation and properties of the globin and globin caseinate that we have used. As in our previous studies, antigenic properties were sought for, first, by the reaction of anaphylaxis, and, secondly, by the test for fixation antibodies in the sera of animals that had been repeatedly injected with one or the other of the substances under consideration.

ANAPHYLAXIS EXPERIMENTS.

Guinea pigs, weighing from 300 to 400 gm., were given subcutaneous injections of 1 c.c. of a 1 per cent. solution of globin (in N/40 hydrochloric acid) or of globin caseinate (in N/40 potassium hydrate), and after a three weeks' interval were injected with one or the other of the substances in the doses indicated and with the results noted in table I.

The anaphylaxis experiments show the toxicity of globin for normal animals. Globin apparently causes symptoms and lesions in the guinea pig in all respects similar to anaphylactic intoxication by a non-toxic protein-like horse serum, or a substance like peptone. Globin, however, is not antigenic, as determined by its property to sensitize for an increased intoxication by itself; guinea pigs that have received an initial dose of globin react no more violently than normal animals to a subsequent dose of the substance (compare guinea pigs 128 and 139, 129 and 141, and 130 and 140).

Globin caseinate sensitized slightly to itself, more markedly for casein, and not at all for globin. Globin also fails to render anti-anaphylactic an animal sensitized to globin caseinate (133 and 134). In other words, the casein alone seems to function in anaphylactic sensitization and intoxication. These experiments, therefore, throw no further light on specificity.

As will be seen, more definite and instructive results were obtained in fixation experiments with the sera of immunized rabbits.

TABLE I.

No. of guinea pig.	Initial injection, subcutaneously.	Injection after three weeks.	Symptoms.
128	1 c.c. of 1 per cent. globin	4 c.c. of 1 per cent. globin intraperitoneally	Irritation and prostration; recovered.
129	1 c.c. of 1 per cent. globin	1 c.c. of 1 per cent. globin intravenously	Dead in 2 min.; emphysema; hemorrhages into lungs and pericardium.
130	1 c.c. of 1 per cent. globin	1 c.c. of 0.2 per cent. globin intravenously	Marked symptoms; recovered.
131	1 c.c. of 1 per cent. globin	4 c.c. of 1 per cent. casein intraperitoneally	No symptoms.
132	1 c.c. of 1 per cent. globin	1.25 c.c. of globin caseinate intravenously	No symptoms.
133	1 c.c. of 1 per cent. globin caseinate	4 c.c. of 1 per cent. globin intraperitoneally. 24 hrs. later 1 c.c. of 1 per cent. globin caseinate intravenously	Irritative and prostrated symptoms; recovered. Doubtful symptoms.
134	1 c.c. of 1 per cent. globin caseinate	1 c.c. of 0.2 per cent. globin intravenously. 24 hours later 4 c.c. of 1 per cent. casein intraperitoneally	Marked symptoms like Nos. 130 and 140. Slight symptoms.
135	1 c.c. of 1 per cent. globin caseinate	1 c.c. of 1 per cent. globin caseinate intravenously. 24 hours later 4 c.c. of 1 per cent. casein intraperitoneally	Doubtful symptoms. Doubtful symptoms.
136	1 c.c. of 1 per cent. globin caseinate	1.25 c.c. of globin caseinate intravenously	Slight symptoms; killed in 1 hr.; gastric hemorrhages.
137	1 c.c. of 1 per cent. globin caseinate	4 c.c. of 1 per cent. casein intraperitoneally	Marked symptoms.
138	Control	1.25 c.c. of globin caseinate intravenously	No symptoms.
139	Control	4 c.c. of 1 per cent. globin intraperitoneally	Marked symptoms like Nos. 128 and 133.
140	Control	1 c.c. of 0.2 per cent. globin intravenously	Marked symptoms like No. 130; recovered.
141	Control	1 c.c. of 1 per cent. globin intravenously	Severe respiratory symptoms; recovered; chloroformed; hemorrhages in lung and lymph node.

FIXATION EXPERIMENTS.

Rabbits were given repeated injections of globin or globin caseinate and were bled a week or ten days after the last injection. The serum was removed the following day, inactivated at 56° C. for one half hour, and used for fixation experiments. An anticasein serum (No. 101 (rabbit)), which was on hand from previous experiments, and another serum (No. 58), somewhat weaker, were prepared in a similar manner.

IMMUNIZATION PROTOCOLS.

Rabbits Treated with Globin.—Rabbit 55 was given four intraperitoneal injections of a 1 per cent. solution of globin in doses of 20, 10, 10, and 5 c.c., and was bled ten days later. Distinct symptoms of prostration followed each injection, from which, however, the animal recovered entirely.

Rabbit 56 was given one intravenous injection of 1 c.c. of a 1.6 per cent. solution of globin in N/40 hydrochloric acid followed by three intraperitoneal injections of 20, 10, and 5 c.c. Bled ten days later. Symptoms the same as in rabbit 55.

Rabbits Treated with Globin Caseinate.—Rabbit 60 was given four intraperitoneal injections of a 2 per cent. solution of globin caseinate in N/40 potassium hydrate in doses of 6, 10, 10, and 10 c.c. at intervals of four, three, and ten days, and was bled six days later. The animal showed marked symptoms of prostration with polypnea after each injection, but recovered entirely.

Rabbit 66 was given five intravenous injections of a 1 per cent. solution of globin caseinate in doses of 2 c.c. at intervals of four days and was bled nine days later. The animal showed no distinctive symptoms.

With these sera and globin, globin caseinate, and casein as antigens, repeated fixation experiments were carried out. The technique of the experiments is similar in all details to the one we have described previously. It may be noted that two preparations of globin were tested (5) and the globin was tried both in acid and in alkaline solutions (both N/40). The solutions of globin caseinate and casein were prepared in 0.85 per cent. salt solution containing N/40 potassium hydrate. A summary of the fixation results is given in table II. Complete fixation means absence of hemolysis after two hours at 37° C. and standing over night in the ice box.

A study of the results leads to the following conclusions in respect to the comparative antigenic properties of globin, globin caseinate,

and casein: 1. Globin is non-antigenic. 2. Globin caseinate gives rise to an antiserum that reacts with itself and almost equally well with casein. The antiserum also gives a definite fixation reaction with globin. 3. Casein gives rise to an antiserum that reacts with casein and almost equally well with globin caseinate. The chief point of interest is the fact that globin when combined with casein becomes antigenic.

TABLE II.

Fixation Experiments with Globin, Globin Caseinate, and Casein and Various Antisera.

Antigens, 1 per cent. solution, in doses from 0.1 c.c. down.	Minimal fixing dose with various antisera employed in doses of 0.3 c.c.[1]						
	Antiglobin serum 55.	Antiglobin serum 56.	Antiglobin caseinate serum 60.	Antiglobin caseinate serum 66.	Anticasein serum 101.	Anticasein serum 58.	Normal rabbit serum.
Globin I (acid solution)	Negative	Negative	Complete, 0.1 c.c.	Partial, 0.1 c.c.	——	Negative	Negative.
Globin I (alkaline solution)	Negative	Negative	——	Complete,[2] 0.05 c.c.	——	——	Negative.
Globin II (acid solution)	Negative	Negative	Partial,[2] 0.01 c.c.	Partial,[2] 0.02 c.c.	——	——	Negative.
Globin caseinate (alkaline solution)	Negative	Negative	Complete, 0.001 c.c.	Complete, 0.01 c.c.	Complete, 0.001 c.c.	Partial, 0.1 c.c.	Negative.
Casein (alkaline solution)	——	——	Complete, 0.001 c.c.	Complete, 0.02 c.c.	Complete, 0.0001 c.c.	Partial, 0.1 c.c.	Negative.

An investigation as to the possible multiplicity of antibodies in the antiglobin caseinate serum immediately suggested itself. It seemed possible that the antiserum might contain separate antibodies for globin and for casein, and to investigate this possibility absorption experiments were resorted to. The results were only partially conclusive owing to technical difficulties.

[1] The dose of alexin fixed is 1 c.c. of a 10 per cent. solution of fresh guinea pig serum. The hemolytic unit added is 1 c.c. of a 5 per cent. suspension of washed sheep blood sensitized with four minimal hemolytic doses of rabbit anti-sheep serum, usually about 0.001 c.c.

[2] In this instance inhibition to fixation (more or less hemolysis eventually) occurred in higher doses.

Experiment 1.—To 1.5 c.c. of antiglobin caseinate serum 60, diluted with equal parts of sodium chloride solution, was added 0.02 gm. of globin. After remaining at 37° C. for one hour with repeated shaking the mixture was filtered. There was no evidence of solution of the globin. The fixing value of this treated serum was then compared with the same amounts of untreated serum 60, with globin, globin caseinate, and casein as antigens with the results given in table III.

TABLE III.

Fixing Properties of Antiglobin Caseinate Serum 60 before and after Absorption with Globin.

Antigens, 1 per cent. solution, in doses of 0.1 c.c.	Untreated serum.	Treated serum.	Antigens, 1 per cent. solution, in doses of 0.1 c.c.	Untreated serum.	Treated serum.
Globin.....	Complete	Negative	Casein.....	Complete	Complete.
Globin caseinate.....	Complete	Partial			

It is clear from this experiment that the absorption of an antiglobin caseinate serum by globin removes all the fixing property for globin and part of the fixing property for globin caseinate, but none of the fixing value for casein. This seems to indicate the presence in antiglobin caseinate of two antibodies, one of which reacts with globin and one with casein, and both, of course, with globin caseinate. It was unfortunately impossible to prove this conclusively by the reverse absorption experiments with globin caseinate and with casein. This was due to the fact that casein goes almost entirely into solution in serum, and globin caseinate partially into solution, leaving a filtrate which is in itself a fixing combination. Attempts to obviate this solution by adding sufficient N/10 hydrochloric acid to neutralize the serum, with or without subsequent return to normal by the addition of aliquot parts of potassium hydrate after removing the casein, were not successful, as the casein still went into solution in the neutral serum and the antibody for globin, as tested by a control serum neutralized by the acid alone, was apparently destroyed. This latter fact may explain why globin in alkaline solution was better fixed by the antiglobin caseinate serum than when in acid solution.

SUMMARY.

This study of globin and its compound with casein (globin caseinate) shows that globin fails to produce fixation antibodies in rabbits after repeated injections, thus agreeing with our own work

and with that of others with similar histon bodies which are primarily toxic. When globin is combined with casein, however, it gives rise to antibodies that react not only with globin caseinate and casein but also with globin. The antibodies in antiglobin casein serum are apparently separate, one for globin and one for casein. In other words, the change in globin undergone on combination with casein has apparently rendered it antigenic.

We did not succeed in demonstrating the genesis of this new antigenic property by anaphylaxis experiments.

A further investigation of similar and more complex combined proteins is indicated and gives promise of more light on the nature of biological specificity.

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