

ANAPHYLAXIS TO SERUM PROTEINS IN THE GUINEA-PIG

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PART 1. ABSORPTION FROM THE INTESTINE

Since the pioneer work of Van Slyke & Meyer [1912] demonstrated the ready absorption of amino acids from the intestine, experiments on the absorption of undigested protein have been almost entirely lacking. It is assumed that during the course of digestion protein is absorbed in the form of amino acids, and this assumption is based on the ample provision of the appropriate enzymes in the gut. It is not known whether individual proteins are prevented from passing the epithelium without previous hydrolysis, on account of the large size of the molecule or some special property of the cells which rejects foreign protein. To explain certain allergic phenomena in man the absorption of protein in small amounts is conceded, but this is without direct proof, and in any case is usually regarded as a pathological process in certain individuals. There seems no reason why molecular size should of itself limit the absorption of unchanged protein from the intestine.

The present work was undertaken to determine whether horse serum albumin and serum globulin, two proteins which differ markedly in molecular weight and which, as Svedberg & Sjögren [1928] have shown, may be isolated homogeneous as regards particle size, can be absorbed from the small intestine; and if so, whether molecular size has an appreciable influence, enabling albumin to be absorbed more readily than globulin. Since it was not to be expected that these proteins would be absorbed in large amount, the ideal method of approach seemed to be by the application of the anaphylaxis reaction. This method has been used with success by Hettwer & Kriz [1925] and by Hettwer & Kriz-Hettwer [1926] in studying the absorption of blood serum from the small intestine of the guinea-pig sensitized by serum. These workers injected serum into loops of intestine of anaesthetized animals and noted whether symptoms of anaphylactic shock occurred. Desensitization was also tested by subsequent intraperitoneal injection of serum. We have carried out upwards of two hundred experiments on anaphylactic shock in guinea-pigs,

giving the reacting dose by intracardiac injection into the unanaesthetized animal, and concluded that the symptoms of shock shown by the animals are somewhat unreliable. If death occurs in a few minutes there can be little doubt, but short of this, misleading conclusions may be reached in many cases.

For the quantitative experiments which were planned in the present work it seemed that the sensitized animal could be used to greater advantage by employing females only, removing one uterine horn and then injecting the protein. While absorption was taking place the first horn could be tested for sensitivity by the method of Dale [1912]. The second horn could then be removed after an appropriate interval and similarly tested. This method has the disadvantage that only females can be used, but the much greater advantages that the initial sensitivity or otherwise of the animal is established, and that the response of the isolated uterus is more reliable than are the symptoms of the whole animal.

MATERIAL AND METHODS

Horse serum albumin was crystallized by the method of Young [1922], twice washed and twice recrystallized by the method of Adair & Robinson [1930]. The crystals of albumin were drained as completely as possible on porous tiles, dried over sulphuric acid and ground to powder. The ammonium sulphate content of the albumin preparations varied from 11 to 15%.

Whole globulin was used, since the work of Svedberg & Sjögren [1930] has shown that euglobulin and pseudoglobulin are very unhomogeneous in solution and are artefacts derived from the parent globulin. The globulin was prepared by precipitation from serum by the addition of an equal volume of saturated ammonium sulphate. After filtration for 5-6 hr. the globulin precipitate was dissolved in a volume of water equal to half that of the serum used. An equal volume of ammonium sulphate was added and filtration was carried out after 5-6 hr. The globulin was precipitated and redissolved four times in all, then the precipitate was drained on tiles, dried and ground. The ammonium sulphate content of some of the globulin preparations was much higher than that of the albumin, and to avoid large differences which would involve troublesome calculations in dosage, all globulin preparations were rejected which had an ammonium sulphate content higher than 20%. Those preparations which were used contained 12-20% ammonium sulphate. When allowance for the salt content had to be made, the ammonium sulphate content of both albumin and globulin was taken to be 15%, a sufficiently close approximation. All figures for the albumin and globulin dosage given are the weights of the dry powder, and no account of the ammonium sulphate is taken unless specifically stated. Sensitization was by intraperitoneal injection of 2 mg. albumin or globulin in 0.1 c.c. 0.9% NaCl. An interval of 2-4 weeks, usually 19-21 days, was allowed in the case of globulin, but, since

Dale & Hartley [1916] had shown that a longer time is needed for albumin sensitization, 4–5 weeks was allowed for the latter. The animals were weighed just before operation, and we have worked always to the nearest 20 g. body weight; an imaginary standard animal of 300 g. or 15 ‘units’ has been adopted, and the weights of protein necessary for injection, and many calculations, have been made on this basis. Only for publication are the weights of protein recorded as per 100 g. body weight.

When sensitization should have been effected, the animal was anaesthetized with ether, the abdomen was opened, and two ligatures were placed on one uterine horn. Two threads were inserted in the muscle between the ligatures and the piece of muscle was removed and set up in the bath, the contractions being recorded on smoked paper. Latterly, the two ligatures were dispensed with; some little time was saved and no significant amount of blood was lost. After the first horn had been removed, the protein was injected into the animal. The wound was then sewn up and the animal was allowed to recover from the anaesthetic. After the desired interval a portion of the second horn was removed, usually after killing the animal by a blow on the head, but often under anaesthesia in the living animal. It has been impossible to detect any interfering action of the ether throughout the experiments. Except where otherwise stated, in testing the first and second horns for sensitivity 0.1 c.c. of whole horse serum was added to the bath, whether the animals were sensitized to albumin, globulin, or serum; this was more convenient and economical of material. The muscle bath contained 120 c.c. of Ringer-Locke’s solution: temperature was maintained at about 37° C. by the burner and copper rod figured by Dale & Laidlaw [1912]. After several hundred experiments in which Ringer solutions were used with the normal and half the normal quantities of calcium at different times [see Dale, 1912], a preference for the latter was established and this has been used throughout the latter half of the work.

RESULTS

Absorption of globulin and albumin from the intestine

The highest possible level in the small intestine was chosen as the site of injection, since during life there would be little chance of undigested protein reaching the lower levels, owing to the ample provision of digestive enzymes. If it could be shown that protein could pass through the upper part of the small intestine when introduced artificially, then it might be conceded that this process may occur under more normal conditions. The protein was ‘dissolved’ in normal saline, no attempt being made to bring the globulin into more complete solution by means of alkali. In these and subsequent experiments no volume of fluid greater than 1.0 c.c. was injected into intestine or blood vessels unless otherwise stated. Injection was made into the first

oop of the duodenum, about 4 cm. from the junction with the pylorus, and $3\frac{1}{2}$ -4 hr. were allowed to elapse before the second horn was removed. No attempt was made to ensure that the stomach was empty; the experiments were all performed in the morning, and the animals had not been fed since the previous afternoon. Experiments on the absorption of albumin and globulin were carried out concurrently, and as the work progressed it was found not only that the animals could be desensitized by the absorbed protein, but that desensitization was effected at exactly the same level in the two series. No symptoms of anaphylactic shock were observed in any of these animals. In Tables 1 and 2 it is shown that 6.0 mg. of each protein per 100 g. body weight were required to desensitize the animal; below this level a response was still elicited from the second horn. Each sign (+ or -) gives the result of a single experiment; the + sign indicates a response of the second uterine horn to the antigen, and the - sign no response, or desensitization.

TABLE 1. Animals sensitized by globulin. Globulin injected into the duodenum

mg. globulin per 100 g.	Reaction of 2nd horn	mg. globulin per 100 g.	Reaction of 2nd horn
4.0	+	6.0	- - - -
5.0	+ + + + +	7.0	- - -
5.5	+ +	7.5	- - +

TABLE 2. Animals sensitized by albumin. Albumin injected into the duodenum

mg. albumin per 100 g.	Reaction of 2nd horn	mg. albumin per 100 g.	Reaction of 2nd horn
5.0	+ + +	7.5	+ - -
5.5	+ + - +	10.0	- + - -
6.0	+ - - -		

Though the same amount of albumin and globulin per unit of body weight was required to desensitize the second uterine horn in the albumin- and globulin-sensitized animal respectively, it could not be said that the two proteins were being absorbed at the same rate unless the same quantities of protein were necessary in the two animals when the antigen was introduced directly into the portal circulation. It was to be expected that the quantity of protein required to desensitize would be higher when injected into the intestine than when given into the blood; the alteration of some of the protein in the gut was almost inevitable. This problem was first studied in the guinea-pig sensitized by albumin. After removal of the first horn, albumin in normal saline was injected into the large vein which runs along the caecum near to the point where the small intestine enters. Cessation of bleeding from the puncture was usually rapid: the intestines were then returned to the abdominal cavity and the wound was sewed up. The second horn was tested for sensitivity $1\frac{1}{2}$ -2 hr. later. The results are given in Table 3 and show that

desensitization was effected when the dose of protein was above 4.0 mg./100 g. body weight. No symptoms of anaphylactic shock were observed in any experiment.

TABLE 3. Animals sensitized by albumin. Albumin injected into the portal blood

mg. albumin per 100 g.	Reaction of 2nd horn	mg. albumin per 100 g.	Reaction of 2nd horn
0.5	+++	4.0	++
1.0	+++	5.0	--
2.0	+++	6.0	-----

*Désensitization of the globulin-sensitized animal by globulin
injected into the portal circulation*

This series was started, as in the case of the corresponding series with albumin, by injecting a small amount of globulin into the mesenteric vein and gradually increasing the dose in succeeding experiments. In these animals severe shock, sufficient to cause death in 3-5 min. after injection of the protein, was met with for the first time. After the death of three animals, as recorded in Table 4, it was clear that though the whole dose might be given in one injection without a fatal result, there was a grave risk of death, and attempts were made to reduce this risk by spreading the dose over two or three injections. This was first done by giving a small amount of protein after removing the first horn, waiting 5 min., and then injecting the second. The third injection, in which the remaining protein was given to bring the whole amount to the desired total, was given 1½-2 hr. later after anaesthetizing the animal and reopening the abdomen. Guinea-pigs stand a second operation very well, and even a third if necessary. When it was thought that an initial dose of 1.0 mg./100 g. could be safely given without shock, two animals given this amount died with typical symptoms, gasping respiration and passage of urine, 3 min. after the injection. The results to this stage are given in Table 4,

TABLE 4. Animals sensitized by globulin. Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
.	.	1.0	++	No reaction of animal
.	.	2.0	++	No reaction of animal
.	.	2.5	++	No symptoms in one animal; the other died in shock 4 min. after injection
.	.	3.0	+	No reaction
1.75	1.75	3.5	+	1st horn removed: 1st injection given: severe symptoms. 2nd injection 5 min. later. Good recovery
1.5	1.5	4.5	++	1st horns removed. Globulin given in three injections at 5 min. intervals. Severe shock in both animals after 1st injection. Good recovery
1.0	.	7.0	++	1st horns removed. 1st injection given. No reaction. Given 2nd injection 1½ hr. later. No reaction
1.0	.	.	++	Severe shock and death of both animals about 3 min. after injection

and show that no desensitization of the second horn was effected by the maximum dose then reached, 7.0 mg./100 g.

The remarkable finding that a globulin-sensitized animal was not desensitized by an injection into the mesenteric vein of an amount of globulin which would desensitize if given by the intestinal route, coupled with the tendency of these animals to die in severe shock after the intravenous injection, suggested that the paradox was not a true one, and was due to the comparatively crude attempts at desensitization when the protein was injected into the blood. It will be recalled that desensitization of the globulin-sensitized animal by globulin injected into the intestine was effected with no symptoms of shock; this was due presumably to the steady slow absorption of the protein, quite different from the sudden influx of the most carefully graded injections. It was argued that the effect of injected antigen on some sensitive cells in the wall of the liver vessels might cause severe spasm of these vessels and so slow down the blood flow through the liver as to allow time for the injected globulin to be absorbed and inactivated, if not digested, by cells in the liver. It is well known that the liver can metabolize amino acids, and it was possible that it might be highly active towards whole protein and change it in such a way that, if sufficient time were allowed, the antigenic power of the protein would be abolished. It will be shown subsequently that there is such absorption and inactivation by the liver of albumin injected into the portal circulation. That the liver must play a more important part than has yet been realized, in the events which follow the injection of the reacting dose of protein into the sensitized guinea-pig, is clear from the death of some of these animals which resulted from the injection of globulin into the mesenteric vein, without desensitization of the uterus: presumably the antigen was held in the liver and some reaction took place there of sufficient severity to kill the animal.

The question whether the failure to obtain desensitization of the uterus in the globulin-sensitized animal by a dose of globulin into the mesenteric vein greater than that required by the intestinal route was due to spasm of the liver vessels, was tested in the following manner. Into the duodenum was injected an amount of globulin which was known to be just insufficient to desensitize the animal (5.0 mg./100 g.). It was thought that after some hours the protein would have passed through the liver in the portal blood, and, though the amount was insufficient to desensitize the whole animal, the liver would have received all the protein, presented in minute amounts spread over a long period, and any sensitive cells there would have been completely desensitized: additional globulin, injected into the mesenteric vein, would then go quantitatively past the cells of the liver which were completely 'saturated' and desensitize the uterus and the whole animal. In these experiments $3\frac{1}{2}$ hr. were allowed for the absorption of the globulin from the intestine; at the end of this time the animal was anaesthetized, the abdomen was reopened, and

the first horn was removed, subsequently to be tested for sensitivity; the globulin was then injected into the mesenteric vein. In the first two experiments 2.5 mg. globulin per 100 g. were given in the second injection: surprisingly, severe reaction occurred in one animal; in neither was the second horn desensitized when the animals were killed $1\frac{1}{2}$ hr. later. In three succeeding experiments, in which in the preliminary injection into the gut 5.0 mg. globulin per 100 g. were also given, 6.0 mg./100 g. were injected into the mesenteric vein. In two animals severe shock and death occurred 4–5 min. after the injection into the blood, and in neither was the uterus desensitized: the first uterine horn of the third animal was found to be insensitive, and in this experiment no symptoms of shock were observed on injection of the protein; this shows that a large dose of globulin into the mesenteric vein is toxic only to an animal appropriately sensitized, a conclusion subsequently borne out by the results of experiments in which even larger amounts of globulin were injected into the portal blood of animals highly sensitized to albumin: in these, as in the non-sensitive guinea-pig injected with globulin, no reaction occurred.

A consideration of the results at this stage led inevitably to the conclusion that the liver of the globulin-sensitized animal must possess a very great capacity to absorb globulin presented to it in the portal blood, and the experiments were continued in which globulin was injected into the animal only by the mesenteric vein, in the certainty that, if the extreme sensitivity of the animal could be overcome by graded injections, ultimately the protein must pass the liver and desensitize the uterus if the total dose was high enough. Since it was known that a small dose of globulin given by the portal route was quite unable to desensitize the uterus, it was thought proper to make the first injection before removing the first horn, and this procedure much facilitated desensitization without shock. The first small amount of globulin was acting while the first horn was being removed; when this had been done, a second injection was given and these two injections could be made while the intestines were exposed for little longer than was required for the ordinary operation of removing the first uterine horn. The wound was then sewed up and the animal was again anaesthetized $1\frac{1}{2}$ –2 hr. later, when the third and final injection was given. No reaction was observed after any injection, and desensitization was effected by the very large dose of 20 mg./100 g. The results are given in Table 5.

The fact that at least three times the amount of globulin required by the intestinal route to desensitize the uterus must be given when the protein is injected into the portal blood may be explained either by supposing that globulin in the intestine is absorbed not as globulin, but as a protein which is reduced in molecular size yet is antigenically indistinguishable from globulin, or that globulin is absorbed from the intestine unchanged, but after passing

TABLE 5. Animals sensitized by globulin. Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
0.5	1.0	10.0	+++	1st injection given, then 1st horn removed. 2nd injection given. Final injection after 2 hr. Killed 2 hr. later. No symptoms of shock
0.5	1.0	15.0	++-	As above, but volume of fluid used in final injections was 1.3-2.0 c.c.
0.5	1.0	20.0	---	2.0 c.c. given in final injections

the mucous membrane enters not the blood but the lymphatic system, thus by-passing the liver. Against the first view is the fact that the antigenic characteristics of proteins are quickly destroyed in the early stages of breakdown. It seems, moreover, doubtful whether such consistent results would have been obtained in the experiments in which animals were desensitized by albumin and globulin injected into the intestine, if absorption were dependent on enzymic breakdown. The crucial test of the second hypothesis would be injection of globulin into the intestine after ligature of the thoracic duct, not an easy operation to perform in the guinea-pig. In support of the second explanation, however, is the remarkable failure to desensitize the liver cells in those experiments in which globulin was introduced into the intestine in amount nearly sufficient to desensitize the whole animal: if the globulin from the intestine entered the blood by way of the lymphatics and the thoracic duct, then the liver cells would not be preferentially desensitized; and when an injection of globulin was given into the portal blood the liver cells might still be capable of such a profound reaction as actually occurred in three animals out of the four tested. The explanation of the death of the animals, in these and other experiments in which the uterus was not desensitized after enough protein had been given by the portal route to kill them, may be found in the work of Dale, Dragstedt and others (for a review see Dragstedt [1941]), in which it was shown that a toxic substance, almost certainly histamine, was liberated from tissues in sensitized dogs and guinea-pigs following the reacting dose of antigen. In our experiments recorded above in which death occurred, presumably histamine was liberated from cells in the liver. Even though the globulin was not in sufficient amount to pass this organ and desensitize the uterus, the liberated histamine might enter the systemic circulation in sufficient quantity to cause death with symptoms very closely resembling those due to generalized anaphylactic shock.

The absorption of albumin and globulin by the liver

At this stage it seemed advisable to assess the capacity of the liver of the globulin-sensitized animal to absorb globulin, and also to determine whether any special affinity was shown by the liver of the albumin-sensitized guinea-pig

for albumin. In these experiments the animal was anaesthetized and the first uterine horn was removed: the abdominal wound was then closed and an incision was made in the neck; protein was then injected into the jugular vein. In the two series of animals increasing amounts of protein were given until the level of desensitization was established. Each animal sensitized to albumin received a single injection of albumin into the jugular vein, and, as in the case of the animals given the protein into the mesenteric vein, no symptoms of shock were observed even when sufficient albumin was given to desensitize the animal. Interaction between albumin and the sensitive cells of the albumin-sensitized animal takes place without shock. Reactions of varying severity, in two cases causing death, occurred when the animals sensitized to globulin received this protein into the jugular vein. Globulin is therefore toxic to animals sensitized by it, whether the globulin is injected into the systemic or portal circulations. Globulin injected into the jugular vein, however, was much more effective in desensitizing the plain muscle of the globulin-sensitive uterus than globulin injected by the portal vein. The results are given in Tables 6 and 7 and show that the amounts of protein required to desensitize the albumin- and globulin-sensitized animals are very similar when given into the systemic circulation: if reference is made to Table 3 it will be seen that there is a large absorption of albumin by the liver of the albumin-sensitized animal, twice as much protein having to be given by the mesenteric as compared with the jugular route to desensitize the uterus.

TABLE 6. Animals sensitized by albumin. Albumin injected into the jugular vein

mg. albumin per 100 g.	Reaction of 2nd horn	mg. albumin per 100 g.	Reaction of 2nd horn
0.25	++	2.0	++
0.5	+++	2.5	--
0.75	+ ~~+~~ +	3.0	--
1.5	++		

TABLE 7. Animals sensitized by globulin. Globulin injected into the jugular vein

mg. globulin per 100 g.		Reaction of 2nd horn	Remarks
1st	Total		
.	0.5	++	All injections under ether. 1½-2 hr. between injections.
0.25	0.75	+++	Animals killed 1½-2 hr. after (final) injection
.	1.5	+	One animal died in shock 3 min. after injection
0.25	1.5	+	Two animals showed moderate reaction after 1st injection
0.5	1.5	--	No reaction
.	2.0	-	Moderate reaction after 1st injection
0.33	2.0	-	No reaction
.	2.5	--	Moderate reaction
0.5	2.5	-	Moderate reaction after 1st injection
			Moderate reaction in two animals: third died in shock 3 min. after injection
			Severe reaction after 1st injection, moderate shock after 2nd

The very large absorption of globulin by the liver of the animal sensitized to this protein was apparently due to the richness of the liver tissues of such

an animal in globulin antibody, and not to some special characteristic of globulin itself. This was shown by injecting globulin by the portal route into animals which had been sensitized by an intraperitoneal injection of 0.1 c.c. of whole horse serum 2-3 weeks previously. This series was started by giving, in graded doses, amounts of globulin approaching those found necessary to desensitize the globulin-sensitized animal. All the early results showed desensitization of the uterus, and the amount was gradually reduced until it was found, not only that globulin passed through the liver of the serum-sensitized animal with remarkable ease, but that the total quantity required to desensitize such an animal was less than one-thirtieth of that needed by the globulin-sensitized guinea-pig when the protein was given by the portal route, and about a third of that required by the globulin-sensitized animal when given by the jugular vein. Desensitization of the serum-sensitized animal was effected by such small amounts of globulin given by the portal route, that it did not seem worth while to perform any experiments in which the protein was given by the jugular route: if there is any absorption of globulin by the liver of the serum-sensitized animal it probably lies within the limits of experimental error of the method. Another fact of interest which emerged as these experiments proceeded was that desensitization must have been effected by the small initial amount of protein used when a large quantity was given by graded doses, yet no symptoms were observed.

Since the serum-sensitized animal required so small an amount of globulin given by the portal route to saturate the receptive cells as compared with the

TABLE 8. Animals sensitized by serum (2-3 weeks). Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
0.5	1.0	12.5	-	No symptoms of shock in any animal. Animals killed 1½-2 hr. after (final) injection
0.5	1.0	10.0	- -	1st injection of globulin given. 1st horn removed. 2nd injection given. Final injection 1½-2 hr. later
0.5	1.0	7.5	- -	Injections as above
0.5	1.0	4.0	- -	Injections as above
0.5	.	1.5	-	Injections as above
.	.	0.5	- - - +	1st horn removed. 1st injection given; 2nd injection 2 hr. later
.	.	0.25	+ - -	1st horn removed. Globulin injected
.	.	0.1	+ + -	1st horn removed. Globulin injected
.	.	0.05	+ + +	1st horn removed. Globulin injected

TABLE 9. Animals sensitized by serum (2-3 weeks). Globulin injected into the duodenum

mg. globulin per 100 g.	Reaction of 2nd horn	Remarks
1.5	- - - +	Animals killed 3½-4 hr. after injection
0.5	- - - +	
0.25	+ + +	
0.1	+ + -	
0.05	+ - - +	

guinea-pig which had been sensitized by globulin, it was of interest to determine whether the amount required by the intestinal route was also smaller. The results of the administration of globulin to serum-sensitized animals by both routes are given in Tables 8 and 9 and show that the amount of globulin which must be injected into the intestine of the serum animal to desensitize it is less than one-tenth of that needed by the globulin-sensitized animal. As might be expected when such small amounts of protein were being used in a biological test of this nature, the results were not sufficiently clear to afford evidence in the serum-sensitized animal as to whether globulin after absorption enters the blood capillaries or the lymphatics, and it is doubtful how much significance must be attached to the two experiments in which desensitization was effected by 0.05 mg. protein per 100 g. given into the duodenum, a level at which desensitization was not effected when the globulin was injected into the portal blood.

SUMMARY OF PART I

1. The absorption of horse serum and of albumin and globulin from the intestine of sensitized guinea-pigs has been investigated. The quantity required to desensitize an animal when the protein was injected into the intestine was compared with that needed when the protein was introduced directly into the portal blood of another animal. The reaction of the isolated uterus was the criterion of desensitization.

2. It is shown that the three proteins, or substances which are antigenically indistinguishable from them, may be absorbed from the intestine.

3. Animals sensitized by albumin require a greater quantity of albumin to be introduced into the intestine to effect desensitization than is needed when the protein is injected into the portal blood. The reverse was found when the desensitization of the globulin-sensitized animal was effected by globulin.

4. The larger quantity of globulin necessary to desensitize the globulin animal when given into the portal blood, compared with that necessary by the intestinal route, has been attributed to fixation of globulin by the sensitized liver.

5. Albumin must be injected into the portal blood of an albumin-sensitized animal in approximately twice the amount required with injection into the jugular vein, to desensitize the uterus; but it is shown that fixation of albumin by the liver of such an animal is much less than that of globulin by the liver of the globulin-sensitized guinea-pig.

6. Fixation of globulin by the livers of sensitized animals appears to be determined by the type of antibody present in the cells.

PART 2. ANTIGENIC CHARACTERISTICS OF BLOOD SERUM

1. Serum as a desensitizing antigen

Nearly forty years ago Hardy [1905] performed experiments which threw doubt upon the dual nature of the protein of blood serum. Having shown that there was no movement of protein in serum when a current was passed through it, Hardy concluded that alkali-globulin could not be present in serum: further, serum could pass through the pores of a clay cell which retained globulin. Hardy was therefore of the opinion that globulin is not present in untreated serum, and suggested that some complex protein breaks up to yield globulin when serum is diluted or precipitated by salts. Since then, so much work has been carried out on the albumin and globulin of blood serum, particularly in relation to their still further subdivision, that the significance of Hardy's original work has been rather lost to sight. A difficulty which was apparent until recently, the implication that in the break-up of serum protein to yield globulin a larger molecule results, has been met by the work of Svedberg & Sjögren [1930], in which it was shown that when globulin breaks up into euglobulin and pseudoglobulin very large particles are formed. Macfarlane [1935], as a result of ultracentrifuge experiments, concluded that serum is unhomogeneous as regards particle size, and while globulin may be present it is not possible to regard the lighter molecular fraction as identical with isolated albumin. The question seems to be incapable of direct proof if any chemical process and even dilution breaks up the supposed serum protein, but it was thought that an investigation into some of the antigenic properties of blood serum might throw light on the problem. The remarkable affinity for globulin of cells in the liver of the globulin-sensitized animal gave a means of approach, by determining the amount of serum required to desensitize when given by the portal route and comparing this with the globulin content of serum as found by analysis. When the literature on the latter point was consulted, wide discrepancy was found in the figures for the albumin and globulin content of horse serum, and though the horse is usually considered to be one of those animals in which the globulin exceeds the albumin in amount, not all authors were in agreement upon this. In work of the nature of that to be undertaken it was desirable to discount small differences and take outside figures: it was assumed therefore that 8 g./100 c.c. would represent the greatest amount of total protein which would be present in any sample of horse serum, and that the albumin/globulin ratio does not differ significantly from unity, i.e. 0.1 c.c. of serum contains not more than 4 mg. of albumin and 4 mg. of globulin.

Desensitization by serum of animals sensitized to globulin

As in the case of globulin-sensitized animals receiving globulin into the mesenteric vein the injection of serum by the same route in similarly sensitized

animals caused the death in shock of some, while others receiving the same dose were unaffected, though the first uterine horns appeared equally sensitive. The same thing was noticed in other series which will be described later and referred to in the discussion. Except when injecting the smallest quantities of serum into the portal blood of the globulin-sensitized animal, the protein was given usually in two doses, the first after removing the first horn, the second $1\frac{1}{2}$ –2 hr. later; the animals were killed $1\frac{1}{2}$ –2 hr. after the second injection. The amount of serum required to desensitize was found to be so small that in order to get below the minimum dose, rather large animals were used and these were given 0.01 c.c. of serum, the smallest quantity which could be measured with any degree of accuracy; from the nature of the problem under investigation any use of diluted serum was prohibited. This accounts for the somewhat curious doses per 100 g. body weight given at the minimum levels. The results are given in Table 10, and it will be noted that death in shock occurred from even 0.01 c.c. of serum, also that 0.0075 c.c. of serum per 100 g. is as effective in desensitizing as 0.1 c.c., or fourteen times as much: at no point above the minimum, up to the maximum dose given, was serum always

TABLE 10. Animals sensitized by globulin. Serum injected into the portal blood

c.c. serum per 100 g.		Reaction of 2nd horn	Remarks
1st	Total		
0.01	0.10	- - +	1st injection given after removal of 1st horn. $1\frac{1}{2}$ –2 hr. between injections. Animals killed $1\frac{1}{2}$ –2 hr. after (final) injection
0.01	0.075	+ - - -	1st injection caused severe reaction in one animal, moderate in others. No symptoms after 2nd injection
0.0075	0.06	-	No reaction in any animal
.	0.05	+ -	No reaction in any animal
.	0.05	+ -	Death 3 min. after injection
0.0075	0.032	-	Death 3 min. after 2nd injection
0.0075	0.02	+ -	Moderate reaction in one animal after 2nd injection
.	0.0075	- + -	No reaction
.	0.0029	-	The last five animals received 0.01 c.c. serum. The last animal died in shock 3 min. after injection
.	0.0023	+ +	
.	0.0020	-	
.	0.0022	+	

TABLE 11. Animals sensitized by serum (2–3 weeks). Serum injected into the portal blood

c.c. serum per 100 g.		Reaction of 2nd horn	Remarks
1st	Total		
0.01	0.05	- + + - -	1st injection given after removal of 1st horn. $1\frac{1}{2}$ –2 hr. between injections. Animals killed $1\frac{1}{2}$ –2 hr. after (final) injection
0.01	0.035	- - +	Severe reaction in one animal after 1st injection
0.005	0.02	+ - -	No reaction in any animal
.	0.015	+	Death 5 min. after injection
.	0.0075	.	Shock and death in 5 min. 2nd horn not examined
.	0.0075	+ - -	No reaction
.	0.0025	+	The last three animals received 0.01 c.c. serum. No reaction
.	0.0023	+	
.	0.0018	+	

effective. Where failure to desensitize occurred, there was no doubt on the matter, a large response was obtained when the antigen was added to the bath. The same phenomenon was observed in the corresponding series where serum-sensitized animals received injections of serum into the mesenteric vein. No explanation can be offered as to why serum is haphazard in its action as a desensitizing antigen.

If the border-line dose of globulin required to desensitize a globulin animal when given by the portal route is taken at 15 mg./100 g., and it is possibly even higher, and allowance is made for the 15% of ammonium sulphate in the globulin used, and the figure is then compared with the globulin content of the amount of serum used for desensitization at the minimal point (0.0075 c.c./100 g.), the ratio is rather greater than 40 to 1. Doubt was at once thrown on the presence of globulin in serum, but it was possible that albumin in serum might be a desensitizing agent in the globulin animal. Though experiments had shown that uterine horns of globulin-sensitized animals showed no response when 10 mg. of albumin were placed in the bath it was possible that there might be some action *in vivo*. Animals sensitized to globulin were therefore injected with albumin through the mesenteric vein, and others received the protein into the duodenum. The converse experiments were performed about this time and may conveniently be taken here; albumin-sensitized animals were given globulin by the portal and intestinal routes. As is shown in Tables 12 and 13, there were no cross-reactions. Though it was now

TABLE 12. Animals sensitized by globulin. Albumin injected into the portal blood and duodenum

mg. albumin per 100 g.	Reaction of 2nd horn	Remarks
6.0	+++	Injection into portal blood. No reaction. Animals killed 2-3 hr. later
6.0	+++	Injection into duodenum. Animals killed 4 hr. later

TABLE 13. Animals sensitized by albumin. Globulin injected into the portal blood and duodenum

mg. globulin per 100 g.	Reaction of 2nd horn	Remarks
8.0	++++	Injection into portal blood. No reaction
6.0	++	Injection into duodenum
15.0	++	Injection into duodenum. Volume of injection fluid was 2.0 c.c.

clear that neither globulin alone, nor albumin alone, could account for the desensitizing power of serum for the globulin animal, the possibility remained that these two proteins in conjunction might effect what neither could carry out singly. Animals sensitized to globulin were therefore injected with a mixture of albumin and globulin ('synthetic serum') by the portal route. The proteins were mixed each day and dissolved in normal saline, the usual concentration being 10 mg. of each protein per c.c. Graded injections were used, the first being given before, the second after removal of the first horn, and the third about 1½ hr. later. When death or severe symptoms occurred

in an animal after the first injection, the initial dose was reduced and desensitization was then effected without difficulty in a number of experiments until a particularly sensitive animal made further reduction advisable. It will be seen from Table 14 that the second uterine horn was regularly desensitized until the level of globulin was below the minimum found necessary when globulin alone was injected into the *jugular* vein, and even at the low level of 0.5 mg./100 g. only two animals out of five were still sensitive. It would seem that the presence of albumin not only allows globulin to pass freely through the liver of the globulin-sensitized animal, but actually reduces somewhat the amount of globulin required to desensitize a given weight of tissue. If the border-line of desensitization by the two proteins combined is taken at 0.5 mg. of each protein per 100 g., a level which is certainly not too low, and the usual allowance is made for the salt content of the protein, there is close agreement with the albumin and globulin content as found by analysis of serum at its minimum desensitizing level in the globulin-sensitized animal.

TABLE 14. Animals sensitized by globulin. Mixture of albumin and globulin injected into the portal blood

mg. albumin and globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
				1st injection given. 1st horn removed. 2nd injection given. Final injection after 2 hr. Animals killed 1½-2 hr. after final injection
0.5	1.0	4.5	--	Severe reaction in one animal after 1st injection
0.25	0.75	4.0	-	No reaction
0.25	.	.	.	Shock and death in 3 min. 2nd horn not examined
0.15	0.6	4.0	--	No reaction
.	.	3.0	-	Severe reaction, but good recovery
0.15	0.6	3.0	-	No reaction
0.15	0.6	2.0	-	No reaction
0.05	0.1	2.0	--	No reaction
0.15	.	1.0	-	Severe reaction. Good recovery. 2nd injection 2 hr. later
0.05	0.1	1.0	- +	No reaction
0.05	0.1	0.5	+ - - - +	No reaction

Desensitization by serum of the animal sensitized to albumin

The efficacy of serum as a desensitizing agent when introduced into the portal blood was investigated in a series of animals sensitized by albumin, with a view to discovering whether this desensitization might be accounted for solely on the known albumin content of serum. It was anticipated that the discrepancy would be even greater than in the case of the globulin animal desensitized by serum, since, unlike the latter animal whose isolated uterus is not responsive to albumin *in vitro*, there is often, but not invariably, some reaction when 5 mg. of globulin are placed in the bath containing the isolated uterus sensitized to albumin. Though no cross-reaction was found *in vivo* when the albumin-sensitized animal was injected with globulin (Table 13), the experiments made *in vitro* suggested that there would be a greater tendency in

this direction than when the converse experiments with globulin-sensitized animals were performed. Such was not discovered: there was found to be a greater desensitizing action on the albumin animal by serum and by a mixture of albumin and globulin than could be accounted for on the albumin content; but the influence of the globulin was much less marked, quantitatively, than was that of the albumin in the converse experiments. The results in this series are given in Table 15. Qualitatively, the action of serum, or of a mixture

TABLE 15. Animals sensitized by albumin. Serum injected into the portal blood

c.c. serum per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
		0.075	-	When two injections given, 1½-2 hr. interval. When three injections, 1st and 2nd given immediately before and after removal of 1st horn. Animals killed 1½-2 hr. after final injection
0.015	.	0.05	- - -	No reaction
0.0075	0.015	0.05	- -	No reaction
0.015	.	0.042	+	No reaction
0.015	.	0.042	+	Death in shock 4 min. after 2nd injection
0.015	.	.	.	Death in 3 min. 2nd horn not examined
0.0075	0.015	0.042	- +	No reaction
0.0075	.	0.042	-	Severe reaction after 1st injection; good recovery. Moderate reaction after 2nd injection. Normal when killed 2 hr. later
0.003	.	0.042	-	Severe reaction after 1st injection; good recovery. Reaction and death 3 min. after 2nd injection
.	.	0.035	++	Severe shock in both animals; death in 3 min.
.	.	0.035	++	Moderate reaction in one animal
0.0075	0.015	0.035	+	No reaction

of albumin and globulin, on the albumin-sensitized animals was very different from that of albumin alone. While no death occurred, or even any sign of shock, when the albumin animal was desensitized by a single injection of albumin, the use of serum or 'synthetic serum' was found to be dangerous to the animal and desensitization had to be attempted by graded doses. Even with the utmost care and the use of a very small initial dose, death occurred in a number of cases when serum was injected: 'synthetic serum' was less toxic. The border-line dose of serum necessary to desensitize was found to be 0.042 c.c./100 g. Below this level the uteri were still sensitive: in the six experiments above this level there was no failure to desensitize, but more observations would be required to make certain that serum does not show its haphazard action in these animals as it does in those sensitized to serum and to globulin. The amount of albumin necessary to desensitize an albumin animal, when given by the portal route, may be taken to be 3.8 mg./100 g. (from Table 3) after correcting for the ammonium sulphate: 0.042 c.c. serum should contain not more than 1.7 mg. albumin. There is thus a large discrepancy in favour of serum, which might be attributed to the contained globulin. Before starting to test the action of synthetic serum on animals sensitized by albumin, it was important to have more exact information as to the amount of albumin required to desensitize these animals when the

protein was given into the portal blood. The series already recorded (Table 3) was adequate for the purpose when the experiments were performed, but, in order to compare the action of synthetic serum with albumin alone in the albumin-sensitized animal, a greater number of results was desirable at the border-line. The extension of these experiments is shown in Table 16, and it will be seen that there is no material difference, the border-line still standing at 4.5 mg./100 g.

TABLE 16. Animals sensitized by albumin. Albumin injected into the portal blood

mg. albumin per 100 g.	Reaction of 2nd horn
3.0	+++
4.0	+++
5.0	---

TABLE 17. Animals sensitized by albumin. Synthetic serum injected into the portal blood

mg. albumin and globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
0.1	0.62	4.0	---	Method of injection as in Table 15
.	.	3.0	-	No reaction
.	.	3.0	-	Death in shock 3 min. after injection
.	.	3.0	+	Severe reaction, but good recovery
0.15	0.62	3.0	---	No reaction
0.15	.	3.0	+	Severe reaction after 1st, none after 2nd injection
0.1	.	2.0	++	No reaction

The experiments with synthetic serum (Table 17) show that the border-line is 3.0 mg. of each protein per 100 g., sufficiently below that with albumin alone to indicate that the globulin did perceptibly augment the desensitizing action of the albumin. The influence of the globulin, however, was much more definite in the production of anaphylactic shock, to which the albumin animals were subject when injected with the synthetic serum, but not at all when injected with globulin or albumin alone. Calculation shows that, in the albumin animal, natural serum is also perceptibly more effective in desensitization than can be accounted for by its content of albumin and globulin; but the difference is small. Altogether, the effect of globulin, in enhancing the desensitizing action of albumin by the portal blood on the albumin-sensitive animal, is far less than that of albumin in enhancing the desensitizing effect of globulin by the same route on the globulin-sensitive animal. To determine whether the globulin in synthetic serum merely abolishes the capacity of the liver cells to absorb albumin, five experiments were performed in which synthetic serum in graded doses was injected into the jugular vein of albumin-sensitized animals, at the level of 2.0 mg./100 g. In two of these the second horn was still sensitive, and comparison with Table 6 will show that in the albumin animal the presence of globulin does little to reduce the absolute amount of albumin necessary to desensitize the tissues.

Desensitization by serum in the intestine

Serum having been shown, when injected into the portal blood, to desensitize albumin and globulin animals at levels which could not be accounted for by its content of albumin and globulin respectively, it seemed advisable to determine whether a similar phenomenon occurred when serum was injected into the intestine, i.e. does serum desensitize an albumin animal from the intestine by virtue of the albumin content as given by analysis, and similarly with globulin for the globulin animal, using for comparison the figures given in Tables 2 and 1? After removal of the first uterine horn, serum was injected into the duodenum of these animals, which were killed about 4 hr. later. When a sufficiently large amount of serum had been given, desensitization was effected, always without any symptoms of shock. Injection of serum into the blood of both albumin and globulin animals may be followed by severe shock and death, but evidently during the slow absorption of serum from the intestine desensitization is sufficiently gradual to obviate any symptoms. The results are given in Tables 18 and 19. In the case of the albumin

TABLE 18. Animals sensitized by albumin. Serum injected into the duodenum

c.c. serum per 100 g.	Reaction of 2nd horn
0.66	- + - -
0.5	- - + +
0.33	- + + +
0.16	+ + + +

TABLE 19. Animals sensitized by globulin. Serum injected into the duodenum

c.c. serum per 100 g.	Reaction of 2nd horn
0.66	- + - -
0.33	+ - - +
0.16	+ - + +

animal it will be seen that the border-line dose of serum is about 0.5 c.c./100 g., corresponding to 20 mg. of albumin. In Table 2 the minimum desensitizing dose of albumin by the duodenum is 6.0 mg., less than one-third. This shows that, while serum injected into the blood is a more efficient desensitizing agent than can be accounted for by its albumin content, the position is reversed with injection into the intestine. A similar result was obtained when serum and globulin were injected into the intestine of the globulin animal, though the difference was less marked. Here the border-line dose of serum is about 0.33 c.c./100 g., corresponding to a globulin content of 13.2 mg., while globulin alone by the intestine desensitizes at 6.0 mg./100 g. The desensitization of the serum-sensitized animal by serum in the intestine was also studied: again, desensitization was effected without symptoms of shock, but the amount required was much greater than in the albumin and globulin animals, and the discrepancy as regards the globulin was enormous. The results given in Table 20 show that the border-line dose of serum is about 0.87 c.c./100 g.,

TABLE 20. Animals sensitized by serum. Serum injected into the duodenum

c.c. serum per 100 g.	Reaction of 2nd horn	Remarks
1.25	- - -	Animals sensitized 15-20 days
0.95	- - -	Animals sensitized 30-35 days
0.80	+ + +	Animals sensitized 15-20 days
0.66	+ + + +	Animals sensitized 30-35 days
0.33	+ + + +	Animals sensitized 30-35 days
0.16	+ + + -	Animals sensitized 15-20 days

corresponding to a globulin content of 34.8 mg., whereas the serum animal (Table 9) was desensitized by 0.5 mg. globulin per 100 g. The very marked difference shown by serum as a desensitizing agent when given by the intestine, as compared with its action when injected into the blood, suggests either that the serum protein is a complex, in which albumin and globulin have no independent existence, and that the cells of the intestinal mucosa will pass this complex protein only with difficulty, or that albumin and globulin, which together make a more efficient desensitizing agent than either alone when injected into the portal blood, impede one another in their passage through the wall of the intestine. Interference might also be caused by the lipins in serum, delaying the passage of albumin and globulin through the intestinal mucosa, though this factor would not account for the great difference between the globulin and serum-sensitized animals, the latter, which required the smaller amount of globulin, needing the larger amount of serum in the intestine. The problem was first investigated in the globulin-sensitized animal. Serum was shaken three times with an equal volume of ether, the ether extracts were combined, measured, and portions were evaporated to dryness in a series of basins which thus contained lipins from a known amount of serum. For use, the lipid residue was rubbed up with a measured quantity of normal saline which was added to that in which the albumin and globulin were dissolved. The mixture was then injected into the duodenum after removal of the first uterine horn, and the animals were killed 4 hr. later. An amount of globulin was chosen which was more than sufficient to desensitize the globulin animal, and the same weight of albumin was also present: the mixture contained lipins from approximately 0.3 c.c. serum per 100 g., that amount of serum which was the border-line dose for desensitization. From Table 21 it will be seen that desensitization was not effected, nor was it

TABLE 21. Animals sensitized by globulin. Mixture of albumin and globulin, with and without lipins, injected into the duodenum

mg. albumin and globulin per 100 g.	Reaction of 2nd horn	Remarks
7.5	+ - + +	Protein and lipins
7.5	+ + + +	No lipins

effected when the albumin and globulin were injected without lipins; the latter can have contributed nothing to the result. Albumin seems to have an inter-

fering action on the passage of globulin through the intestine of the globulin-sensitized animal. This result became all the more difficult to explain when it was found that a similar effect was not observed in experiments on the albumin and serum-sensitized animals. Lipins were not added, since the result of the experiments on the globulin-sensitized animals could not be attributed to them. There was no interference when albumin and globulin together were absorbed from the intestine of the albumin and serum animals. Serum seems to behave as a unique desensitizing agent when operating from the intestine.

TABLE 22. Animals sensitized by albumin or serum. Mixture of albumin and globulin injected into the duodenum

mg. albumin and globulin per 100 g.	Reaction of 2nd horn	Remarks
7.5	- - + -	Animals sensitized by albumin
7.5	+ - - -	Serum-sensitized animals (15-20 days)

2. Serum as a sensitizing antigen

Though the experiments described in the preceding section threw doubt on the separate existence of albumin and globulin in blood serum, none was conclusive. It remained to study the antigenic properties of serum from the opposite direction, and determine how far these might agree with those exhibited by albumin and globulin together, when acting as sensitizing antigens. When an animal which has been given an injection of serum first becomes sensitive, the isolated uterus is responsive to globulin, not to albumin: this was shown by Dale & Hartley [1916], and was confirmed on the first uterine horns of the animals used in Table 8. No reaction to 5 mg. albumin was observed, but if 1 mg. of globulin was placed in the bath a large, usually maximal, response was obtained. The uteri of these animals were thus indistinguishable, qualitatively, from those of animals sensitized by globulin. When the animals thus sensitized by serum, at a stage at which only sensitiveness to globulin was present, received an injection of globulin into the portal blood, the animals as a whole were very different from globulin-sensitized animals, since they were readily desensitized, without symptoms, by a small dose of globulin. The difference in the behaviour of the intact animals sensitized by globulin and by serum respectively, the antibodies in whose uteri were responsive only to globulin (or to serum, which causes a reaction in all types but does not differentiate them), could only be attributable to the presence of albumin in the serum, either free or combined with globulin to form serum protein. Experiments were therefore performed to determine whether animals sensitized by a mixture of albumin and globulin behaved like serum-sensitized animals, both as regards the reactions of their isolated uteri and the desensitizing effect on them of injected globulin, when examined under 21 days

from the time of injection, i.e. at a time when the albumin, if acting independently, could not cause any response to this protein in the isolated uterus [Dale & Hartley, 1916]. Seven animals were given an injection of 2.5 mg. albumin and 2.5 mg. globulin in 0.2 c.c. normal saline and were examined 12–19 days later. The first uterine horn of each animal was removed and 0.5 mg. globulin per 100 g. was then injected into the mesenteric vein. One animal died in shock about 4 min. later. The others showed no symptoms, and received a further injection of globulin $1\frac{1}{2}$ hr. later, 1.0 mg./100 g. A total quantity of 1.5 mg. globulin per 100 g. was chosen for these and similar experiments to be described later, since it was felt that any failure of this amount to desensitize the animals would be significant in differentiating the globulin from the serum type of animal (see Tables 4 and 8). No symptoms were observed, and the animals were killed about $1\frac{1}{2}$ hr. later. The second horns were in every case insensitive to 0.1 c.c. serum placed in the bath. The first horns had shown no response to 5 mg. albumin, but after changing the Ringer's solution gave large responses to 1 mg. of globulin. The uteri of the animals sensitized by serum and by synthetic serum are therefore, at this stage of sensitization, qualitatively identical; and the desensitization action on the two groups of globulin introduced through the portal circulation seemed also to be the same, with the exception that in the group sensitized by 'synthetic serum' one died in anaphylactic shock, while none of those sensitized with natural serum has ever shown any symptoms following an injection of globulin. The conclusion seems inescapable that albumin and globulin given at the same time act in conjunction as sensitizing antigens to give rise to a sensitized animal apparently identical with one which has received serum, up to 19 days from the time of injection: the albumin in the animal receiving synthetic serum could not be shown to have influenced the isolated uterus, but it had influenced certain receptive cells, presumably in the liver, so as to allow a small amount of globulin to pass and to desensitize the uterus.

Another series of animals was sensitized by albumin and globulin, 2.5 mg. of each protein, but the animals were kept for a longer period and were examined between the 30th and 35th days. The first uterine horns were indistinguishable from those of the previous series; there was no reaction to albumin, a large response to globulin. Differences, however, appeared in the reactions of some of the animals as a whole, as may be seen from Table 23. Two animals died in shock as a result of the second injection, even though a small amount of globulin had been injected $1\frac{1}{2}$ hr. previously. Of the eleven animals which survived and which were killed $1\frac{1}{2}$ –2 hr. after the final injection, four were not desensitized. This suggests that the influence of the albumin given in the sensitizing injection had waned, and that these four animals were becoming like those sensitized to globulin alone. In the remaining seven animals the influence of the albumin had persisted, and these were indis-

TABLE 23. Animals sensitized 30-35 days by a mixture of albumin and globulin.
Globulin injected into the portal blood

mg. globulin per 100 g.		Reaction of 2nd horn	Remarks
1st	Total		
.	1.5	+	1st injection after removal of 1st horn. 2nd injection (when given) 1½-2 hr. later.
.	1.5	-	Animals killed 2 hr. after final injection
0.15	1.5	++	Severe shock and death in 4 min.
0.15	1.5	- - + - + - + - -	No reaction
			Death in shock 3-4 min. after 2nd injection
			No reaction

tinguishable from those animals examined 12-19 days after sensitization with synthetic serum.

It remained to examine a number of animals sensitized by natural serum 30 days and over, to determine whether there was any difference between them and the 15-20 day serum animals as regards the reactions of the isolated uteri and of the animals as a whole. Twelve such animals were used. After removal of the first horn an injection of globulin was given into the mesenteric vein, 0.15 mg./100 g.; 1½ hr. later the second injection was given, bringing the total to 1.5 mg./100 g. The animals were killed 1½-2 hr. later. All the animals survived, with no sign of shock after the first or second injections: the uteri of all were desensitized. As regards the whole animal therefore, there is no difference between this series and those sensitized only 15-20 days by serum. Nor could any difference be detected in the first uterine horns of the 30-35 day serum-sensitized animals; they did not react to albumin placed in the bath, but gave large responses to globulin. This finding conflicts with that of Dale & Hartley [1916] who showed that the uterus of the serum-sensitized animal 28 days and upwards, unlike the 15-20 day animal, is responsive *in vitro* to a small amount of albumin: these workers also found that, after a larger quantity of albumin had been added to the bath and the sensitivity of the uterus to this protein had been entirely discharged, the muscle still responded to euglobulin. Though the volume of Ringer's solution used in our experiments was larger, no explanation is possible on the lines of a lower concentration of albumin. The difference is essentially qualitative: Dale & Hartley found the uterus of the 31 day serum-sensitized animal more sensitive to albumin than to euglobulin, and stated: 'There was some indication that concurrently with the late development of sensitiveness to albumin, the sensitiveness of the plain muscle to the globulins, which attains its maximum somewhere between the 14th and 21st day, undergoes some decline, to judge by the size of the initial dose needed to produce a pronounced reaction.' In our experiments 1 mg. of albumin caused no response, nor did 5 mg., but after changing the Ringer's solution there was a large, usually maximal response to 1 mg. of globulin. The serum-sensitized animal appears to be 'stable'

both as regards the reactions of the isolated uterus, and the response of the whole animal to injected globulin, from the 15th to the 35th day, thus differing sharply from the animal sensitized by a mixture of albumin and globulin which is 'unstable'. Experiments were undertaken to determine whether the serum-sensitized animal is stable over a longer period. A series of animals received an intraperitoneal injection of 0.1 c.c. serum and was examined between the 49th and 51st days. Eight animals were used; in no case was there any response of the isolated uterus to albumin, but the usual large reaction followed the introduction of globulin to the bath. Four of the animals received an injection of globulin, 0.15 mg./100 g., after removal of the first horn, and in one a severe reaction followed, but the animal recovered and was normal $1\frac{1}{2}$ hr. later, when this animal, with the others, received the second injection of globulin to bring the total to the standard figure of 1.5 mg./100 g. When the animals were killed $1\frac{1}{2}$ –2 hr. later the uterine horns were all sensitive to globulin. The other four animals received the usual preliminary injection of 0.15 mg. globulin per 100 g. and no shock was observed. In the second injection a larger amount of globulin was given to bring the total to 5 mg./100 g.; one animal suffered from severe shock after the second injection but made a good recovery. When the animals were killed $1\frac{1}{2}$ –2 hr. later, all were found to be desensitized. The serum-sensitized animal is thus not stable indefinitely, but requires, with increase of time from the date of sensitization, an increased amount of globulin to discharge the sensitivity.

Sensitization by diluted serum

The differences observed between animals sensitized 30–35 days by serum and by synthetic serum might have been due to the quantities of albumin and globulin in the latter being present in a different ratio from that in which they exist in serum. It would not necessarily imply, therefore, that albumin and globulin are present in serum in some type of combination differing from one which might be formed when albumin and globulin are remixed after separation. Sensitization of animals by diluted serum would throw light on this question, since, if serum contains a complex protein, the albumin and globulin which can be isolated from it must be dissociated at some stage by simple dilution of the serum, or by the addition of a salt such as ammonium sulphate, or, possibly, by the operation of both factors. Serum diluted 1/10 with water and with normal saline was used. The dilutions were made in the morning and the serum was allowed to stand until the afternoon at room temperature. After stirring, 1.0 c.c. was injected into each guinea-pig and the animals were examined between the 30th and 35th days. The reactions of the first horns *in vitro* were the same as those of all the other 30–35 day serum or synthetic-serum animals; there was no response to albumin, a large response to globulin. After removal of the first horns 0.15 mg. globulin per 100 g. was injected into

the portal blood; 1.0 mg. globulin per 100 g. was injected $1\frac{1}{2}$ hr. later. The animals were killed after $1\frac{1}{2}$ –2 hr., with the exception of one which died in shock 4 min. after the second injection. One animal showed a severe reaction after the first injection, but made a good recovery. Reaction in only two animals may not be significant by itself to differentiate this series from those animals which were sensitized by undiluted serum, but the second uterine horns of four of the twelve animals were not desensitized. All of the twelve animals sensitized with injections of natural serum given 30–35 days previously were desensitized by such globulin injections, and none showed any symptoms of shock in the process. The different results observed after sensitization with diluted serum, in which all the constituents of the natural serum were present in the sensitizing dose, in the same quantities and proportions, and the only change made was the previous dilution of the dose, suggest that the difference was due to the breaking up of some complex in which the proteins exist in the natural, undiluted serum, so that the resulting products gave rise to sensitized animals similar to those sensitized with 'synthetic serum' and kept for the same period (cf. Table 23).

TABLE 24. Animals sensitized 30–35 days by diluted serum.
Globulin injected into the portal blood

mg. globulin per 100 g.		Reaction of 2nd horn	Remarks
1st	Total		
0.15	1.5	—	Severe reaction after 1st injection, but good recovery. Serum diluted with NaCl
0.15	1.5	+	Death in shock 3 min. after 2nd injection. Serum diluted with NaCl
0.15	1.5	++--	No reaction. Serum diluted with NaCl
0.15	1.5	+- - - + -	No reaction. Serum diluted with water

Sensitization by separated injections of albumin and globulin

The complete suppression of sensitivity to albumin in the isolated uteri of animals sensitized by a mixture of albumin and globulin was so remarkable as to merit further investigation. While the non-appearance of albumin sensitivity in the serum-sensitized animals might have been due to the absence of free albumin from untreated serum, no such explanation was possible in the animals sensitized by 'synthetic serum'. Guinea-pigs were therefore sensitized by an injection of 2 mg. of globulin and, after 19 days, when full sensitivity to globulin would have been established in the cells, 2 mg. of albumin were injected into the animals. A further period of 30–33 days was allowed to elapse and the animals (which for brevity will be referred to as G-A animals) were then examined. The first uterine horns of eight of the sixteen animals were removed before any globulin was injected, and when the reactions of these uteri to albumin and globulin had yielded consistent results it was considered permissible to give a small preliminary injection of globulin to the

remainder before removing the uteri, with a view to aiding desensitization without death in shock: the G-A animals were found, indeed, to be very susceptible to globulin, and death often occurred. Of the eight first uterine horns examined, one showed no response to albumin or to globulin; in another there was a maximal response to 1 mg. of albumin and none to globulin: the former had failed to become sensitized to either protein, the latter had evidently not been sensitized by the globulin, and the cells had been left free to acquire albumin sensitivity. The remaining six uteri showed no response to 1 mg. of albumin: 5 mg. were then added with no result: the Ringer's solution was changed and a large response to 1 mg. of globulin occurred in each case. The uteri of the eight succeeding animals, removed after the first injection of globulin, all responded to 1 mg. of globulin, but showed no response to 5 mg. of albumin. In fourteen animals the uteri were responsive to globulin alone and therefore were indistinguishable from those of animals which had been sensitized only by globulin. The results of the globulin injection (Table 25)

TABLE 25. Animals sensitized by albumin 19 days after sensitization by globulin (G-A animals). Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
First uterine horns removed before injection of globulin				
.	.	0.5	+	Severe shock; death in 3 min.
.	.	0.15	+	Severe shock; death in 3 min.
0.15	.	1.5	+	2nd injection 1½ hr. after 1st. Moderate shock. Animal recovered, then became weak and died ¾ hr. later
0.15	.	1.5	+	Severe shock after 1st injection. Severe shock after 2nd injection 1½ hr. later; death in 3 min.
0.15	.	1.5	+ -	No reaction. 1½ hr. between injections
First injection of globulin before removal of 1st horn. 2nd injection given immediately after; final injection 1½-2 hr. later				
0.15	0.35	1.5	- -	No reaction
0.05	0.35	1.5	- - -	No reaction
.	.	0.15	.	Death 3 min. after injection
0.05	0.10	1.5	.	Death 5 min. after final injection
0.05	0.10	1.5	.	Death 15 min. after final injection
2nd horns of last three animals not examined				

show that the animals differed from animals sensitized by an injection of globulin only, since, of the seven animals which survived the usual $1\frac{1}{2}$ -2 hr. period after the second injection, all but one were desensitized. The injected albumin therefore had influenced the whole animal; though it was not detectable by the reaction of the first uterine horn, it had affected certain other sensitized cells, probably in the liver, in such a way that they allowed a sufficient amount of globulin to pass them without fixation, and so to desensitize the plain muscle elsewhere. The G-A animals were also much more difficult to desensitize without death in shock than were animals which had been injected only with globulin. A few experiments were performed to determine whether the albumin could affect the globulin antibody in the

G-A animal, at a time when it could not cause an antibody to be fixed in the cells if it were the only protein injected, i.e. whether albumin in the G-A animal influenced the globulin antibody directly or indirectly after formation of albumin antibody. Five animals were available which had been injected with globulin 19 days previously; an injection of albumin was given and the animals were tested after a further 10 days. Globulin, 0.15 mg./100 g., was injected into the mesenteric vein, the first horn was removed, and 0.35 mg./100 g. was injected. One and a half hours later globulin was given to bring the total to 1.5 mg./100 g. When the animals were killed after the usual period the uterine horns of two were found to be desensitized. The result was inconclusive, and a larger number would be required to settle the point: no sign of shock was observed in any of these animals.

The converse of the above type was examined, animals which were given an injection of albumin and after 31 days were injected with globulin (A-G animals): these were killed 17-20 days after the globulin had been given. The first uterine horns of six were removed before any globulin was given into the mesenteric vein, and were tested with 1 mg. of albumin, then with an additional 1 mg. of albumin; the Ringer's solution was changed and 1 mg. of globulin was placed in the bath, followed by 5 mg. Two uteri were insensitive to albumin and to globulin: the remaining four responded by maximal contractions when 1 mg. of albumin was introduced; a further 1 mg. of albumin caused no reaction, nor did globulin. The whole of the sensitivity of these uteri was thus discharged by a small amount of albumin. The remaining five animals received a small injection of globulin before the first horns were removed, since the A-G animals seemed to be even more sensitive to globulin than was the G-A series: the uteri, however, all responded to albumin, none to globulin. None of the isolated uteri of these animals was tested with globulin before introduction of albumin into the bath, since it was vital to be certain of the sensitivity of these uteri to albumin, and thus to differentiate them from the uteri of animals sensitized by serum, synthetic serum, and globulin followed by albumin. The results of the injection experiments are given in Table 26. In two of the animals, death in typical anaphylactic shock occurred about 5 min. after an injection of globulin. Three others, which showed no immediate reaction to the injected protein and which made good recovery from the operation, gradually collapsed and died $\frac{1}{2}$ -1 $\frac{1}{4}$ hr. later. Only four lived for the standard period of 1 $\frac{1}{2}$ -2 hr., and two of these animals were noticeably weak. Globulin appears to cause delayed and fatal shock in the A-G animal, while it caused no symptoms of any kind when injected into the animal sensitized by albumin alone. This fact of itself is sufficient to indicate that, though the uteri of the A-G series are highly sensitized to albumin, the animals are quite different from albumin-sensitized animals. Also, all the second uterine horns which were examined were found to have

TABLE 26. Animals sensitized by globulin 31 days after sensitization by albumin (A-G animals). Globulin injected into the portal blood

mg. globulin per 100 g.			Reaction of 2nd horn	Remarks
1st	2nd	Total		
First uterine horns removed before injection of globulin				
0.15	.	1.5	--	No reaction. 1½–2 hr. between injections. Animals killed 1½ hr. after 2nd injection
0.15	.	1.5	–	Animal died in severe shock 5 min. after 2nd injection
0.15	.	1.5	–	Gradually became weak and died 1 hr. after 2nd injection
First injection of globulin before removal of first uterine horn; 2nd injection immediately after; final injection 1½–2 hr. later				
0.15	0.35	1.5	–	Severe shock after 2nd injection. No shock after final injection, but was weak when killed 1½ hr. later
0.05	0.10	1.5	–	No reaction; very weak when killed 1½ hr. after final injection
0.05	0.10	1.5	–	Died 1½ hr. after final injection
0.05	0.10	1.5	.	Died ½ hr. after final injection; 2nd horn not examined
0.10	0.20	.	.	Severe shock and death 5 min. after 2nd injection. 2nd horn not examined

been desensitized: no response was observed when 1 mg. of albumin was placed in the bath. The sensitivity of the A-G animals to albumin had all been discharged by a small amount of globulin. These facts must indicate that the sensitizing dose of globulin, though it had failed to establish any sensitivity in the uterus which could not be discharged by albumin, had nevertheless influenced the albumin antibody so that it was neutralized by globulin *in vivo*. Since it is difficult to conceive of entirely different actions of globulin *in vitro* and *in vivo*, it is also highly probable that had the first uterine horns been tested by the introduction of globulin into the bath before albumin, a maximal response would have been obtained to the former protein, and that the sensitivity would all have been discharged by globulin. More experiments will be required to be certain on this point, but a consideration of the different series of animals sensitized, viz. by serum, 'synthetic serum', globulin followed by albumin, and albumin followed by globulin, must lead to conviction that in all these animals both albumin and globulin had actively participated in antibody formation; the differences exhibited in the reactions of these animals and of their uteri show that the antibody cannot have been the same in every case. Albumin and globulin must influence one another mutually in antibody formation, but the type of antibody which results is dependent upon the conditions under which the proteins are presented to the animal.

DISCUSSION

The uteri of 797 animals were examined. Of these, 665 had been injected previously with 5 c.c. of milk in the routine test for tubercle carried out in the Department of Bacteriology, for which they were kept 4-5 weeks before being killed. The injection of albumin, globulin, or serum was given to these animals at the appropriate time after the injection of milk, so that the date

of killing should be correct for both the milk test and the anaphylactic work. The high degree of species specificity of the anaphylactic reaction precluded any interference by the proteins of milk, but the validity of results obtained from animals in which tuberculosis was active was open to doubt. After consideration of a long series of experiments carried out on the milk-test animals, it has been decided not to exclude results from animals which had been infected by the milk. All the animals used appeared healthy when the operations were performed, and only when the records of the Department of Bacteriology were consulted, at intervals of about 6 months, were the results of the milk tests known. On looking back through the work performed during such a period, it was not possible to detect any anomalous results which could be attributed to the presence of tuberculosis. The disease did not significantly affect the capacity of the animals to become sensitized to horse protein. Of the 368 animals which were sensitized, 37 or 10.1 % were tuberculous: of the 297 animals whose first uterine horns proved to be insensitive, 26 or 8.7 % had tubercular lesions. The average weight of the milk-test animals was 368 g. These animals were not necessarily virgin, and some were found to be pregnant when the abdomen was opened: if pregnancy was not advanced these uteri were used, many with success, since, if the strip of muscle relaxed to give a steady series of uniform small contractions, the large response which followed the introduction of the antigen into the bath was as clearly marked as in the case of a small virgin uterus.

In addition to the milk-test animals, 132 small virgin guinea-pigs were specially obtained for the anaphylactic experiments alone; their average weight was 298 g. These animals were used late in the work, not because of any doubt as to the validity of the other results, but to supplement the supply of animals from the Department of Bacteriology at a time when it was inadequate, to confirm results which differed from those of other workers, and when it was necessary to sensitize some animals for longer than 4-5 weeks.

After the experiments were completed, there appeared an important paper by Cohen [1943], on the effect of dry grinding on the properties of proteins. Cohen, working with ovalbumin, showed that grinding the protein in a dry state for 24 hr. or longer, produced changes in the molecule associated with denaturation and coagulation. It seems possible that some alteration in the proteins may have occurred during the grinding of the albumin and globulin used in our experiments; the time of grinding was not controlled, and doubtless varied in different preparations, but it was never prolonged. When the work of Cohen was known, the time of grinding of three globulin preparations was accurately noted; globulin was chosen, since this protein becomes a hard glass involving more grinding than albumin. The longest time taken to reduce the protein to the usual degree of fineness was 1 min. 25 sec., and it is doubtful whether any preparation used in this work was ground for more than 2 min.

The very large difference in the time of grinding in our experiments and those of Cohen, suggests that the extent of the denaturation of the albumin and globulin from this cause must have been very small. Appreciable denaturation would reduce the value of conclusions drawn from the difference found between serum and the proteins derived from it, when injected into the circulation. On the other hand, denaturation would enhance this difference when the intestinal route was being used; under these conditions serum was shown to be much less active than albumin and globulin.

The primary purpose of this work, the decision as to whether the smaller molecule albumin is absorbed from the intestine more readily than globulin, has not been achieved. The unexpected finding that globulin may be absorbed, probably by the lymphatic system, renders valueless any calculation based on the relative amounts of the two proteins which are required to desensitize the animal when injected into the portal blood. There is no reason why albumin itself may not enter the lacteals after leaving the lumen of the intestine; but there is not even indirect evidence that this may occur, as there is in the case of globulin. All that can be said, until further information is available, is that albumin and globulin, or proteins antigenically indistinguishable from them, are absorbed in significant amounts within a few hours of their introduction into the small intestine. Such absorption is probably not limited to the artificial conditions which obtained in this work, and it may well occur normally if some protein escapes the action of the proteolytic enzymes in the digestive tract. There was no significant distension of the gut when the albumin and globulin were injected, since the volume of fluid did not exceed 1.0 c.c. When serum was used to desensitize the animals sensitized by this protein, desensitization was only effected by an amount so large that it was impossible to avoid marked distension of the duodenum. Since the gut was not ligatured the distension would be gradually relieved as the serum distributed itself, but it is impossible to be certain how far the distension factor came into play, a factor which Hettwer & Kriz [1925] have shown to affect the absorption of serum from the intestine.

The fact that some sensitized animals died in anaphylactic shock, while others, receiving the same sensitizing treatment and reinjection, showed no symptoms, is not easy to explain. In the animal sensitized to albumin and desensitized by a single injection of this protein, the antigen-antibody reaction must be a comparatively mild event in the cells, and, if it involves some cellular injury with liberation of histamine, the amount so liberated must be small. When such animals were desensitized by serum or synthetic serum a much more drastic event occurred, and there was always the risk of death, even when the antigen was given in graded amounts. Other examples of highly toxic reactions have been given in the text and need not be repeated here. It is remarkable that it was possible, on occasion, to desensitize an

animal by a full dose of the appropriate antigen without shock, while other animals of the same series showed symptoms or died after only a minute dose. The first uterine horns of the animals which were desensitized without shock were often as sensitive as those of animals to which a much smaller dose of antigen proved fatal. It is possible that the reaction of certain sensitized cells in the liver may afford an explanation. The possibility of the active participation in anaphylactic shock in the guinea-pig of sensitized cells elsewhere than in the lungs was considered by Dale [1912], who concluded that there was no necessity to assume any other than the immediate effect of the antigen on the sensitized muscle in the lungs, to explain acute shock. Numerous instances have been given in the present work of sudden and rapidly fatal shock in some sensitized guinea-pigs, particularly in those sensitized to globulin, which follows injection of the antigen into the portal blood, without accompanying desensitization of the uterus; it is probable therefore that the liver may play an important part in some acute anaphylactic reactions in these animals. When fatal shock occurred in 3-5 min. after injection of an antigen into the portal blood, and the uterus was found to be fully sensitive, very little, if any, of the foreign protein could have entered the systemic circulation, and death must have been caused indirectly: the spasm of the bronchial musculature, which is usually considered to be the immediate cause of death in anaphylactic shock in the guinea-pig, must have been secondary to the liberation from the liver of some non-antigenic stimulant of smooth muscle, such as histamine. It is possible therefore that when animals are sensitized by a protein, receptive cells in some tissues, e.g. the liver, are more highly sensitized than in others, and that this differential distribution of the sensitizing antibody varies from one individual to another. This may account for the death in shock of some animals given a very small amount of protein, by the portal circulation, and the absence of any symptoms in others, of apparently equal uterine sensitivity, which received a far larger quantity of the antigen. Protein introduced into the intestine enters the circulation so slowly as to desensitize even the most highly sensitive cells without symptoms, for none have been noted in all the animals desensitized by this route.

It is known that the epithelial cells of the intestinal mucosa discriminate sharply between substances of similar physical properties, and the failure to correlate, in the three types of sensitized animals studied, the desensitizing power of serum with that of albumin and globulin, is a point in favour of serum containing a complex protein in which albumin and globulin have no separate existence. The properties of serum as a desensitizing antigen, when injected into the portal blood, agree closely with those found for an artificial mixture of albumin and globulin, with the curious exception that serum was less consistent in its action than was 'synthetic serum' injected into the globulin animal. Globulin alone was also more reliable than serum in desensitizing

the animal sensitized by the latter. A mixture of albumin and globulin injected into the blood was found to desensitize in circumstances which could not be explained as the action of either of the proteins alone, and it is difficult to account for this in any other way than as due to some type of recombination between them. Macfarlane [1935] has shown that mixtures of albumin and globulin show changes in the ratio of large to small molecules, which must be due to some interaction. If protein complexes exist in serum and in mixtures of albumin and globulin, they are unlikely to be identical, since definite differences were found between serum and 'synthetic serum' when acting as desensitizing antigens. There was also a difference between them when acting as sensitizing antigens. The most cogent evidence in favour of serum containing a complex protein was afforded by the special properties of the guinea-pig which had been sensitized by serum: these were not always reproduced when 'synthetic serum' was used, nor were they always exhibited after sensitization by prediluted serum. The evidence that serum is unique as a sensitizing complex is essentially based on the influence of the 'masked' albumin, an influence which leaves the uterine horns unresponsive to albumin *in vitro*, but which makes the serum-sensitized animals differ completely from those sensitized to globulin, and remain so for at least 35 days. That the serum antibody should show evidence of breakdown between the 35th and 50th days is not incompatible with serum being an albumin-globulin complex, for this must be of the loosest possible kind: it is reasonable to suppose that the antibody may be dissociated ultimately by factors in the cells, just as the antigen which gave rise to it is readily dissociated by water or salt solutions. The alteration which takes place in the serum antibody is compatible with waning influence of the albumin component, which results in the animal becoming more like one sensitized to globulin. A similar change takes place in the animals sensitized by synthetic serum, but at an earlier date. Since nothing is known of the composition of these antibodies, it is not profitable to speculate further, but the results of the experiments with the G-A and A-G animals suggest that there may be fixed in the cells of different animals more than one type of antibody, in the formation of which both albumin and globulin have taken part. An animal sensitized by a single protein can have only one type of antibody present in the cells, and this should remain unchanged, qualitatively, until it disappears in the course of time; when an antibody complex is formed as the result of the injection of two proteins, the components need not necessarily persist with the potency of each unchanged over a long period; one may diminish in strength before the other.

The non-homogeneity of serum shown by Macfarlane [1935] in his ultracentrifuge experiments is not incompatible with serum containing a protein complex. As Macfarlane stated, there was no evidence that the large particles are globulin, and it is possible that there may be more than one type of protein

complex in serum. If albumin and globulin associate together, complexes may be formed in which the constituent proteins are present in different proportions, thus giving rise to particles of different size. Other difficulties are the change from normal of the albumin/globulin ratio in pathological sera, and the analytical figures which show different rates of regeneration of these proteins in the blood after haemorrhage. It is possible that serum protein is not of fixed and invariable composition, and that it may be made up of different proportions of the constituent proteins, depending upon the conditions. It is not easy to see how a final answer to this question may be given; from the very nature of the problem the evidence must be indirect. All that can be said at the present time is that serum, examined by different observers and different methods, does not behave merely like a solution of free albumin and free globulin.

SUMMARY OF PART 2

1. Considerable quantities of globulin have been administered by the intestinal and portal routes to guinea-pigs sensitized by albumin. Cross-reactions did not occur, nor were they observed when the converse experiments were performed.
2. Though the amount of globulin required to desensitize a globulin-sensitive animal by the portal route was large, a very small quantity of globulin was found to be effective when an equal amount of albumin was present ('synthetic serum'). The volume of natural serum required to desensitize such an animal was also very small.
3. The characteristics of serum and 'synthetic serum' as desensitizing antigens have been studied and compared. 'Synthetic serum' simulates serum closely in some reactions, but not in all.
4. The influence of serum as a sensitizing antigen has been investigated. Animals sensitized by serum for 30 days differ from some of those sensitized by diluted serum or by a mixture of albumin and globulin.
5. Animals which were sensitized by serum for 50 days differed from those which were examined after 30 days.
6. The behaviour of serum as a desensitizing and as a sensitizing antigen is incompatible with that of a mixture of albumin and globulin, and it is suggested that serum contains a protein complex in which albumin and globulin have no separate existence.
7. The isolated uteri of animals sensitized by serum, 'synthetic serum', or globulin followed at 3 weeks' interval by albumin are not responsive to albumin. The uteri of animals sensitized by albumin followed by globulin after 31 days are very sensitive to albumin *in vitro*.
8. It is shown that more than one type of antibody may exist, in the formation of which both albumin and globulin have taken part.

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