EXPERIMENTS ON THE PRODUCTION OF SPECIFIC ANTISERA FOR INFECTIONS OF UNKNOWN CAUSE.

III. The Effects of a Serum Precipitin on Animals of the Species Furnishing the Precipitinogen.

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It has been shown previously that specific antisera for infections of which the exciting agent is unknown can be produced in some instances by the use of infected tissue itself as antigen; and that when such sera have been detoxified they can be used successfully for therapeutic purposes.[1] The toxicity is due to the presence of antibodies elicited by the tissue component of the antigen, which, needless to say, are highly injurious to animals of the species furnishing said antigen. It is easy to remove the more obvious of these injurious elements—hemolysins and hemagglutinins—by their selective absorption with red corpuscles, as was done in our type experiments. The precipitins we then encountered were weak and without recognizable action on the animal organism. Whether strong precipitins can cause damage has remained for determination, as has the point of how such damage could be avoided. The problem is not without practical significance in its relation to the utilization of the serum of infected human individuals as antigen.

The study of anaphylaxis has led to the development of a large and complex literature on the effects of the union of precipitin and precipitinogen upon an animal organism to which both are foreign; and a number of papers have been published on the results of injecting precipitinogen into animals which have developed a circulating precipitin. But there exist few observations, and these more or less casual, regarding the effects of a precipitin upon animals against

[1] Rous, P., Robertson, O. H., and Oliver, J., *J. Exp. Med.*, 1919, xxix, 283.

whose serum it is specifically effective. This alone concerns us here. Uhlenhuth and Haendel[2] state in a foot-note to other matters that guinea pigs show severe, anaphylaxis-like symptoms after the intraperitoneal injection of 0.5 to 1 cc. of an anti-guinea-pig rabbit serum with a precipitin titer of 1 to 20,000. The same serum in amounts of 0.75 to 2 cc. caused death. They give a few protocols suggesting tolerance to a second injection. Doerr and Moldovan[3] made closer observations. They state that the precipitating anti-guinea-pig rabbit serum which they employed had *in vitro* a slight hemolytic activity for guinea pig cells, no more than that of normal rabbit serum, yet 2 cc. intravenously killed guinea pigs within 2 hours, while 1 cc. gave rise to severe symptoms, and 0.5 cc. to dyspnea and slight symptoms. Following an intraperitoneal injection there was heightened resistance to a second injection 24 hours later. The experiments cited are few; and the authors make no mention of the presence or absence of hemagglutinins, nor, it seems, did they look for evidence of *in vitro* hemolysis. These are important points for the interpretation of their work, as will be shown.

Removal of Hemolysins and Hemagglutinins.

It is well known that immunization with blood serum as antigen leads to the development not only of precipitins but of hemolysins and hemagglutinins, even though care has been taken to render the serum free of formed elements. In our first experiments along the lines of the work just described it became evident that these antibodies were seriously to be reckoned with. Hemolysis was never noteworthy on *in vitro* tests, being practically absent when guinea pig complement was employed, and in this regard our sera corresponded with the serum of Doerr and Moldovan. Yet these same sera, inactivated, gave rise *intra vasam* to a profuse breaking down of red cells, as evidenced by hemoglobinuria, hemoglobinemia, extreme anemia, spodogenous spleen, and free blood pigment in the fluid of the body cavities. Some fatalities were manifestly attributable to this cell destruction combined with hemagglutination. The latter phenomenon was always pronounced both *in vitro* and *in vivo*. That death can be due to it alone is well recognized. The blood of the animals sometimes showed an almost massive clumping. In view of all these findings the fact that the precipitating sera gave rise, as they did, to the more or less sudden death of guinea pigs was not surprising.

[2] Uhlenhuth and Haendel, *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 761.

[3] Doerr, R., and Moldovan, J., *Z. Immunitätsforsch., Orig.*, 1910, vii, 223.

In order to free the sera of hemolysins and hemagglutinins resort was now had to selective absorption with guinea pig red cells. Our technique for this has been described in a previous paper.[1] Care was taken to keep the serum sterile and to free it of all possible stroma fragments by prolonged centrifugation prior to injection. Also, it was overabsorbed, that is, exposed to far more guinea pig red cells than were enough to remove the demonstrable antibodies for these elements. Our experience,[1] like that of others, has been that the strong hemolytic and hemagglutinative serum resulting from immunization with red cells is completely deprived of toxicity when thus treated. Such was far from being the case with the precipitating serum now in question. After absorption it retained the major part of its toxicity, giving rise to sudden death almost as often, in almost the same dose, and with the same symptoms as when untreated save for inactivation. But there was the difference that all lesions referable to hemolysis and hemagglutination were now lacking. Thus a number of late fatalities were avoided. Manifestly, from these results, Doerr and Moldovan were correct in supposing that the toxicity of their serum was due to another element than hemolysis.

Removal of the Precipitin Does Not Remove Toxicity.

What is this other element? The guinea pig cells used in the absorptions were freed of serum by careful and repeated washing in "gelatin-Locke's" solution, which keeps these usually fragile cells intact. The rabbit serum repeatedly incubated with several successive portions of them remained clear and almost free from hemoglobin. Friedemann[4] has shown that mixtures of red cells and hemolysin in the presence of complement may yield a toxic body before any hemolysis occurs. But this does not happen when complement is absent, as was regularly the case in our work. The possible influence of traces of the washing solution can be ruled out on the basis of previous experience. It seemed likely that the precipitin content of the serum, which was not lessened by repeated absorptions, constituted the toxic element. And in line with this idea, though not necessarily evidence for it, was the fact that the toxicity of different serum specimens

[4] Friedemann, U., *Z. Immunitätsforsch., Orig.*, 1909, ii, 591.

varied in general with their precipitin titer. Attempts were made, therefore, to detoxify the sera by the removal of their precipitin content through specific precipitation. The precipitin was readily removed. But to our great surprise the sera remained as toxic as before.

Specimen Experiments.

Experiment 1.—Two rabbits which had received five intraperitoneal injections of guinea pig serum at intervals of 6 days were bled to death from the heart 10 days after the last, and the serum was at once pooled, inactivated, and tested for hemolysin and hemagglutinin. The undiluted serum caused a faint trace of hemolysis when incubated for 2 hours with equal parts of a 5 per cent suspension of guinea pig red cells and a 1 in 10 dilution of fresh guinea pig serum. In such mixtures agglutination was massive with quarter strength serum and was faintly seen with a 1 in 32 dilution of it. The injection of 2 cc. of the serum into the ear vein of a 200 gm. guinea pig[5] was followed in a few minutes by sneezing, restlessness, severe dyspnea, and complete prostration, with slow recovery during the next 12 hours.[6] The urine for some hours after the injection contained much hemoglobin.

50 cc. of the inactivated serum was now incubated under aseptic conditions with four successive portions of guinea pig red cells, twice washed in a large excess of gelatin-Locke's solution. The portions consisted of 7.5, 7.5, 9, and 6 cc. of packed cells respectively, and the period of incubation ranged from ½ to 3 hours. No agglutination was observable in the last two serum-cell mixtures, and *in vitro* tests showed the complete absence of hemolysin. The absorbed serum, when injected into two guinea pigs of 225 and 215 gm. weight, in amounts of 2 and 1.35 cc. respectively, gave rise to exactly the same symptoms as the unabsorbed, though they were somewhat less severe. The urines of both animals remained free from hemoglobin.

The precipitin titer of the serum was now taken in mixtures of a constant amount of antibody with decreasing antigen, so as to avoid solution of the precipitate in an excess of the latter. By the use of a blood-counting pipette, previously standardized with mercury, as a measuring chamber, small amounts of the undiluted sera were mixed as such. Precipitation occurred in mixtures up

[5] For the technique of such injections see Rous, P., *J. Exp. Med.*, 1918, xxvii, 459. The operation is rendered more simple and certain if the ear is fixed on a ground glass platform, instead of the opaque one previously described, and transillumination is employed.

[6] 3 to 4 cc. of normal rabbit serum can be injected into the circulation of a 200 to 250 gm. guinea pig without the production of symptoms (see Friedberger, E., *Med. Klin.*, 1910, vi, 510).

to and including that containing 5,120 parts of rabbit serum to 1 from the guinea pig, and in 20,000 to 1 when the antigen was diluted to a constant bulk with salt solution after the usual method. The greatest precipitate, coarsely floccular, was seen at approximately 80 to 1 of the whole sera. Accordingly the bulk of the remaining rabbit serum was mixed with sterile guinea pig serum in this proportion, incubated 2 hours, left in the cold over night, and centrifuged until free from the several cubic centimeters of precipitate. It now failed to cause any clouding in mixtures with guinea pig serum above 16 to 1, yielded a slight cloud at 8 to 1, a dubious trace at 4 to 1, and none at 1 to 1. Yet this same serum, injected intravenously into three guinea pigs of 225, 205, and 225 gm. in amounts of 2.2, 1.45, and 1.35 cc. respectively, killed the first animal in 3 hours and 8 minutes, the second in 5½ hours, and in the third gave rise to a moderate "shock" with symptoms resembling those of anaphylaxis.

The results suggest that the serum was rendered, if anything, more toxic by the repeated absorptions.

It is known that a slight, slow precipitation takes place in pooled precipitin sera from different individuals of the same species; and Friedberger[7] has shown that precipitating mixtures will, in the presence of complement, yield a toxic product *in vitro*. To rule out this possible factor in the results, our tests were repeated with individual rabbit serum; and when several sera were to be pooled they were often subjected beforehand to a separate inactivation. In neither case was any difference noted in the results. In the experiment which now follows the sera were inactivated immediately after pooling, and subjected to precipitation with guinea pig serum prior to the absorptions with red cells.

Experiment 2.—The sera of four precipitin rabbits were pooled, inactivated, the precipitin titer was taken, whole guinea pig serum being used, and on the basis of the findings most of the pooled serum was submitted to an optimum precipitation. This was all done on the same day, as rapidly as possible. The optimum precipitation occurred in a 90 to 1 mixture with undiluted guinea pig serum, but clouding was noted in mixtures up to and including 5,120 to 1. The untreated serum injected intravenously into two guinea pigs of 325 and 400 gm., in amounts of 1.65 and 1.8 cc. respectively, killed the first mentioned animal within ¾ hour and caused great, though brief, prostration of the second. 1.85 cc. of the treated serum killed a 275 gm. guinea pig in 1 hour, and 2 cc. caused moderate symptoms in a guinea pig of 375 gm. All four animals had hemoglobinuria.

[7] Friedberger, E., *Z. Immunitätsforsch., Orig.*, 1909–10, iv, 636.

Both the treated and untreated sera were now submitted to five successive absorptions with twice washed red cells in the proportion of 23 cc. of serum to 2.3, 2.3, 2.3, 4.05, and 3.8 cc. of packed red cells. The contact periods ranged from 1 to 2 hours. Agglutination, which in the first mixture was well marked, diminished to a trace in the last one—an exactly similar trace for both the "precipitated" and untreated serum, as *in vitro* tests showed. No hemolysin could be found in the test-tube, with guinea pig complement. 1 part of salt solution was now added to 90 of the unprecipitated specimen, and comparative intravenous injections were carried out with it and with the precipitated serum (Table I).

TABLE I.

Serum absorbed only.			Serum absorbed and precipitated.		
Weight of animal.	Amount injected.	Result.	Weight of animal.	Amount injected.	Result.
gm.	*cc.*		*gm.*	*cc.*	
275	2.1	Died in 1 hr., 51 min.	250	2.1	No symptoms.
275	2.0	" " 1 " 5 "	275	1.8	" "
			275	2.2	Very severe shock.

Tests showed that the absorbed and precipitated serum still contained enough precipitin to cause clouding in mixtures up to and including 320 to 1 with whole guinea pig serum. An optimum was found at 20 to 1 and the serum submitted to a new precipitation in this proportion. Thereafter it still gave a moderate flocculation with an equal amount of guinea pig serum, but no clouding in mixtures above 10 to 1, or 16 to 1 when the precipitinogen was diluted with salt solution. Comparative tests *in vivo* were again made. 1 part of salt solution was added to 20 parts of the unprecipitated serum prior to the injections (Table II).

TABLE II.

Serum absorbed only.			Serum absorbed and precipitated.		
Weight of animal.	Amount injected.	Result.	Weight of animal.	Amount injected.	Result.
gm.	*cc.*		*gm.*	*cc.*	
375	2.0	Moderate shock.			
300	1.8	Severe "			
300	2.15	Died in 11 min.	300	2.2	No symptoms.
275	2.2	Severe shock.	250	2.2	Very severe shock.

At every stage in the treatment of the serum, cultures on agar and in bouillon were taken. These remained uniformly sterile.

In this instance, in contrast with Experiment 1, the attempts to detoxify the serum seemed to have some degree of success, and we were encouraged to further trials. These will not be detailed. They showed that individual differences in the test animals were mainly accountable for the wide variations in the results. Some animals were practically unaffected by the serum that killed others; and no matter how thoroughly the serum was exhausted with red cells and freed of precipitin it remained highly injurious. This was true even when it was given locally. For when injected subcutaneously or into the skeletal muscle it produced a severe lesion. But before describing this its general effects will be taken up.

Effects of the Serum Deprived of Precipitin.

In guinea pigs reacting to an intravenous injection of the exhausted and precipitated—or, for that matter, unprecipitated—serum there is a latent period of from 3 to 10 minutes during which the behavior is normal. Then the animal becomes restless, running about, scratching itself, perhaps sneezing, springing into the air, or twitching. The hair roughens, the urine and feces are usually voided, and an inspiratory dyspnea rapidly appears, accompanied in severe instances by cyanosis and complete prostration, followed by death in a few minutes or hours. Occasionally convulsions precede the fatal issue. Often there is only a paresis of the hind legs, or the animal is now prostrate, now on its feet again, and in these instances of milder symptoms recovery may be very rapid. More often, while recovering, the guinea pig sits crouched, cold, and with staring coat for some hours. When handled it is passive and weak. But by the next day recovery seems complete, and further observation proves that it indeed is so.

The animals that succumb show little on gross examination. The lungs may be distended, and the blood fail more or less markedly to clot, as in anaphylaxis; but these are by no means constant findings. There may be fresh petechiæ in the lungs and intestinal mucosa, as so often after violent death of any sort. This is all that is found if the animal has died within a few minutes of the injection. When it has survived for some hours the liver is always greatly congested, and in it there may be observed microscopically the only lesion that is

present with any regularity; namely, an acute central congestion of the lobuli, often with small hemorrhages, and, if death be deferred long enough, areas of necrosis as a result of the latter. There are no gross hemorrhages in the hepatic tissue. It would seem that the lesion might be secondary to an acute stasis in the heart or lungs, were not an abnormal distension of the right side of the heart entirely lacking at autopsy. In animals surviving several hours polymorphonuclear leucocytes may collect in considerable numbers in the pulmonary capillaries, but in those dying early this feature is absent. The blood shows no hemagglutination and its serum no trace of hemolysis.

The local lesion that follows a subcutaneous injection of 0.5 to 2 cc. of the serum, treated or untreated, is an edema, widespread about the point of injection, with numerous capillary hemorrhages and small, scattered foci of acute inflammation, which, however, do not go on to purulence. The injury may extend into the muscle. A broad, edematous, red-purple patch beneath the skin, with yellowish, ill defined, little, opaque patches and points scattered here and there throughout it, is characteristic. The acute inflammation is at its height at the end of 48 hours, but resolution occurs rapidly thereafter. Cultures remain sterile throughout. Necrosis such as the serum of cattle causes in guinea pigs[8] is never noted. The ordinary hemolytic and hemagglutinative serum obtained by the repeated injection of washed guinea pig corpuscles into rabbits, causes in guinea pigs a lesion somewhat similar in the gross to the one with which we are concerned; but there is this important difference, that the extravasated red cells which render the patch purple are for the most part hemolyzed so that few are seen on section, and those few are agglutinated into clumps. Furthermore, we have found, as already stated, that absorption with red cells rids such a serum of its ability to cause local lesions other than such slight ones as normal rabbit serum may mechanically produce.

[8] Uhlenhuth and Haendel, *Z. Immunitätsforsch., Orig.*, 1909, iii, 284.

Parallel Experiments on the Dog.

The work was now repeated on dogs, since for practical purposes it was important to learn whether our findings were peculiar to guinea pigs. The serum employed came from a number of rabbits repeatedly injected with dog serum. The precipitin titer was not so high as in the case of anti-guinea-pig serum, and hemolysins and agglutinins were no stronger, yet per kilo of animal it proved nearly as toxic. The toxicity was unimpaired by absorption with four portions of red cells followed by a single, unusually successful precipitation which removed practically all the precipitin. The intravenous injection of only 2.7 cc. per kilo of the sterile, exhausted and precipitated, or unprecipitated, serum regularly caused marked symptoms in dogs, and resulted once in death after 18 hours. A latent period of 5 to 10 minutes always followed the injection, and then the animal showed sudden signs of weakness, staggering and lying down, or standing unsteadily with drooping head. Vomiting and defecation took place, and soon there was complete prostration, the dog lying on its side in a semiconscious condition. The respiration was not disturbed, but there was an enormous drop in blood pressure, so that the pulse could no longer be felt and sometimes the heart beat was scarcely palpable. In most cases prostration did not endure more than 1 hour, after which recovery was rapid and complete. The animal which succumbed had received absorbed but unprecipitated serum. The single lesion found was similar to that observed in guinea pigs but more pronounced, being a destructive hemorrhagic congestion of the central half of the liver lobules. The blood in the large vessels, which failed to clot, showed no trace of hemagglutination, and the right side of the heart was not greatly distended. The hemorrhages into the liver substance were far more numerous and pronounced than in guinea pigs and this was the case as well with the lesions resulting from local injection in dogs, which otherwise did not differ from those in guinea pigs, save that edema was sometimes less marked. The numerous fine capillary hemorrhages in the edematous tissue at the site of injection often coalesced to give the appearance of a gross extravasate. Normal rabbit serum, old and new, in the amounts here dealt with, failed to cause any local or general disturbances.

Relation of the Phenomena to Anaphylaxis.

The "shock" produced by the serum will be seen from our description to resemble strikingly in both guinea pigs and dogs that called anaphylactic; but on close analysis points of difference declare themselves. At autopsy the lungs of guinea pigs may not be found distended, though they often are so; and the blood may be clotted. The latent period after an intravenous injection is much longer in dogs than that preceding the anaphylactic paroxysm. More important is the fact that desensitization cannot be effected either by small, graduated injections or by one that results in shock. We have given especial attention to this point, since Doerr and Moldovan present a few protocols which seem to indicate that they succeeded in desensitizing with their precipitating serum, and if this were the case it would offer a way to the safe, therapeutic utilization of our own. But their results must be referable to individual animal variation such as has already been mentioned, for our many tests have definitely shown that even after an injection of absorbed, or absorbed and precipitated, serum which calls forth a severe reaction, there may be no tolerance whatever to a second dose, whether it be given into the blood stream or locally. This is true in dogs as well as in guinea pigs. For example, a dog weighing 4 kilos was given 11.6 cc. of exhausted and precipitated serum into an ear vein. There resulted severe "shock," but with rapid and apparently complete recovery. 4 days later another and similar injection was given, and this called forth exactly the same severe but transient reaction. The animal was killed 6 days after the second injection, and in its liver active repair was found to be taking place of a recent hemorrhagic lesion such as has already been described. When several, small, desensitizing doses or one large one were used (in guinea pigs) the results were no better. The local effect in the guinea pig has some similarity to the Arthus phenomenon, but the latter is in our experience a less severe type of lesion in this species and far more difficult to elicit, at least with horse serum, while it lacks the hemorrhages caused by the absorbed and precipitated rabbit serum.

It is well known that the development of serum sickness is accompanied in man by the appearance of precipitins in the blood, and

that the urine may show a coincident albuminuria. We have followed the urines of a number of dogs and guinea pigs subjected to severe "shock" by the intravenous injection of precipitated or unprecipitated serum which had been exhausted with red cells. None showed noteworthy urinary change. Casts were regularly absent, and the slight trace of albumin occasionally noted was no greater than was inconstantly present prior to the injection.

Despite all this, there is no denying that the effects of the serum may be due to the same toxic principle or principles concerned in anaphylaxis. But, if so, an important difference in the quantitative relations must be assumed.

Possible Sources of the Toxicity.

Is the toxic element primarily present in the serum, or is it engendered by treatment? We feel convinced that the former is the case. The "shock" produced by the absorbed, or absorbed and precipitated, serum differs in no essential from that caused by inactivated but otherwise untreated precipitating serum, as observed by Uhlenhuth and Haendel, Doerr and Moldovan, and ourselves. Were it due to hemolysins and hemagglutinins persisting after absorption, the examination of the blood and the autopsy findings would give evidence of this, while furthermore the process of repeated absorption, even if incomplete, would greatly diminish the toxicity of the serum. Neither is the case. Our serum, as already mentioned, was over-absorbed; that is, submitted to more red cells than were necessary to take out all demonstrable hemolysins and agglutinins, a process which renders completely innocuous the ordinary hemolytic and hemagglutinative serum.

Many observations were made which bore on the question of whether gross precipitation within the animal body might not be a cause of the disturbances noted. Always the precipitated serum failed *in vitro* to cause a clouding when mixed with even a little more than its bulk of guinea pig serum, far less than the preponderant quantity encountered on its injection into the blood. Such tests would seem to rule out actual precipitation as the cause of disturbance, unless indeed the conditions with plasma differ greatly from

those with serum—and there is no reason to suppose that they do, since complement is unnecessary for the precipitin reaction. We have said that the toxicity of the different sera varied in general with their original precipitin titer. Yet that the "shock" engendered by the serum had no essential dependence on the immediate precipitin content was well shown in the results with serum from which the precipitin had been removed. The possibility remains that the toxic element may be a product of the interaction of precipitin and precipitinogen, one formed as readily when the two are brought together without as within the animal body. Against this is the fact shown by Friedberger[7] that specific precipitation *in vitro* fails to give rise to a toxic element unless complement be present; for it was absent in our experiments. But Friedberger made his tests of toxicity on animals of a species to which both precipitin and precipitinogen were strange, whereas in our work the precipitin was directed against the serum of animals of the species used for the tests, and just such serum was employed for the *in vitro* removal of precipitin.

There remains the interesting possibility of the presence in the serum of a hitherto unrecognized toxic antibody. Further work alone can justify any speculation in this direction.

SUMMARY.

There is present in serum of high precipitin titer, produced by the repeated injection of rabbits with the blood-free serum of guinea pigs or dogs, a principle highly toxic for animals of the species furnishing the antigen. Intravenously the serum causes severe shock, and even sudden death, while locally it gives rise to acute inflammatory changes and profuse capillary hemorrhages. The complete removal of hemolysins and hemagglutinins from the serum by exposing it repeatedly to washed red cells lessens its toxicity to only a slight degree and one obviously dependent on these elements; while the further removal of precipitin by specific precipitation *in vitro* has no detoxifying effect whatever. Whether the toxic principle is a hitherto unrecognized antibody or perhaps a toxic product of the interaction of precipitin and precipitinogen,—one formed as readily in the test-tube as in the animal body,—remains to be determined.

The symptoms of guinea pigs and dogs given an intravenous injection of treated or untreated serum markedly resemble those of anaphylaxis, but our attempts at desensitization have been unsuccessful. The local lesion in guinea pigs is more severe than that of the Arthus phenomenon. But these differences from anaphylaxis may, of course, be dependent merely on differing proportions of constituents that are themselves, as yet, scarcely apprehended.

Our observations, as here summed up, were made with a practical point in mind, and as regards this point they are of a discouraging nature. In papers already published it has been shown that sera specifically effective against infections of which the excitant is unknown can in some cases be obtained by using infected tissue itself as antigen. Such sera must, of course, be deprived of antibodies injurious to tissue, prior to their employment in the animal body; and this was successfully accomplished in our early experiments by exhaustion with washed red cells. The purpose of the present work was to determine whether serum used as antigen gives rise to injurious principles in the antiserum. For the serum of infected individuals would in many diseases form a convenient antigen. It is evident that injurious principles result from its use, and that they are not removed from the antiserum when the latter is exhausted with red cells and its precipitin removed by specific precipitation, nor can their action be nullified by desensitization as carried out in anaphylaxis. Unless the obstacle of their presence is in some way overcome the body fluids of infected human beings cannot be practically utilized for the production of antiserum. In test animals the difficulty is not so grave. For we have found that the toxic antiserum produces no enduring lesions when it is administered intravenously in non-lethal doses.