ON THE FORMATION OF SPECIFIC ANTI-BODIES IN THE BLOOD FOLLOWING UPON TREATMENT WITH THE SERA OF DIFFERENT ANIMALS, TOGETHER WITH THEIR USE IN LEGAL MEDICINE.

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During the thirteen years which have elapsed since the publication of my researches upon the bactericidal properties of the blood and other fluids of the body[1], a large amount of most valuable knowledge has been acquired regarding the blood both in health and disease. Through the work of many investigators the complicated subject of immunity is gradually being solved, and we are beginning to understand the way in which the body combats or is protected against the inroads of disease. We have learnt of the existence of specific antitoxic, agglutinative, haemolytic, bactericidal and cellulicidal properties in the blood-serum, etc., as also of a number of neutralizing bodies to these. The quite recent discovery of specific precipitins, which act upon various bacterial products, milks, peptone, egg-albumin, and upon human blood and its derivates, has opened a wide field for investigation, which cannot fail to ultimately yield results of the greatest importance. We have to thank bacteriological investigation for the greater part of the advances which have been made, although a considerable portion properly belongs in the domain of physiology.

[1] Nuttall, G. H. F. (5 July, 1888), Experimente über die bacterienfeindlichen Einflüsse des thierischen Körpers. *Zeitschr. f. Hygiene*, Bd. IV. pp. 353—394. (One plate.)

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Before considering my own experiments regarding the anti-bodies or precipitins produced in the body experimentally I shall briefly describe the work which has been done on the subject. The majority of the papers referred to have appeared during the course of our investigations, which were commenced in January, and we find that we have been able to extend and confirm, in some cases quite independently, the observations of others.

The Action of Specific Precipitins upon certain Bacterial Filtrates.

The existence of specific precipitins in the blood was first demonstrated by Kraus[1] (12 Aug. 1897), who added cholera, plague, and typhoid anti-sera to filtrates of the cultures of the corresponding germs. The bacterial filtrates were prepared by means of stone-filters. On adding an anti-serum to an homologous culture-filtrate a precipitate was formed, but this did not happen if the anti-serum was non-homologous. A filtrate of crushed cholera germs gave the same result as the fluid-culture filtrate, proving that the substances acted upon were contained in the bacterial cell. This accounts according to Kraus, for the results of Widal, Levy and Bruns, who produced immunity by means of cholera and typhoid culture-filtrates, the immunified animals yielding an anti-serum which agglutinated the corresponding germ. On the other hand, a filtrate of diphtheria culture gave no precipitate on the addition of antitoxic horse-serum. These observations were confirmed by Nicolle (March, 1898) who worked with cultures of *B. coli*, *B. typhi*, and *Vibrio massauah*. He obtained most precipitate from old cultures. The reaction was not impeded by the addition of antiseptics.

Tchistovitch states that Marmorek obtained a similar precipitation when he added anti-streptococcic serum to filtered cultures of the *Streptococcus*.

The Action of Specific Precipitins on various kinds of Milk.

We are indebted to Bordet (March, 1899) for the first observations upon the production of specific anti-bodies which act on milk. Having partially sterilized milk by exposing it to a temperature of 65° C., he injected it at intervals, intraperitoneally, into rabbits. After the rabbits had been treated for some time they were bled, and it was found that

[1] See Bibliography at the end of this Paper.

their blood-serum had acquired a specific precipitin which acted only upon the particular milk against which the animal had been immunified. In testing, he placed about 3 c.c. of the anti-serum in a tube; control tubes receiving a like quantity of normal rabbit-serum. Six to fifteen drops of milk were then added to the serum. The mixture with normal serum remained diffusely white, that with anti-serum soon showed granules and subsequently a definite precipitate.

The experiments of Bordet were repeated by Wassermann and Schütze (2 July, 1900 and 29 Jan. 1901), who treated rabbits with human, goat, and cow's milk, obtaining in each case a specific anti-serum. The rabbits treated with human milk yielded a serum which precipitated the casein of human milk, but not the casein of the other milks tested, and *vice versâ*. Schütze (29 Jan. 1901), who published the details of these experiments, dwells upon the hygienic importance of this method in the study of the chemistry of milk, especially nowadays, when efforts are being made to simulate mother's milk. He cites the observations of Fisch (Feb. 1900) in America, the results of the latter being of an analogous nature. Fisch found that an emulsion of udder-cells, injected into animals, gave the same results as when milk was injected, another proof for the theory that milk is not simply a filtration-product of the milk-gland, but a true solution of the cells of that gland[1].

In the experiments of Wassermann and Schütze the rabbits were treated by subcutaneous injections of 10—20 c.c. of milk, repeated every 3 to 4 days. In some cases, doses as large as 30 to 50 c.c. were injected. The milk was sterilized by means of chloroform prior to injection. The animals were bled after 3 weeks, their serum being added in the proportion of 1 : 1, or 1 : 5, to milk diluted 1 : 40; the mixture being left for some hours at room-temperature. Schütze calls the anti-sera produced by milk-inoculations "*Lactosera*." According to Schütze, milk which has been boiled for half-an-hour has lost the greater part of its power to form a precipitate on the addition of its specific lactoserum.

These experiments very clearly show that there are essential differences in the composition of various kinds of milk, and that heating milk alters the composition of the albuminous molecule.

[1] The original paper by Fisch has unfortunately remained inaccessible.

The Action of Specific Precipitins upon Peptone Solutions.

Tchistovitch (May, 1899) treated rabbits with injections of 10% peptone solution, the dose introduced being 5 c.c. He was unable to observe the formation of precipitins in the rabbit's serum. Myers (14 July, 1900), on the other hand, has obtained positive results with solutions of Witte's peptone. Exposed to a temperature of 56° C. for half-an-hour, the anti-serum exerted less action. The weakening due to heat was markedly counteracted through the addition of normal rabbit-serum to the heated serum, although the normal serum had no effect when used alone.

Myers considered that his results strongly support the view that the production of immunity is due to processes of assimilation, peptone disappearing from the blood as does tetanus-toxin. Just as Wassermann showed that certain organ-emulsions neutralize tetanus-toxin, so Neumeister[1] showed that peptone could be neutralized by pieces of intestine. The fact that such heated "pepto-serum," if I may be permitted the expression, could be rendered again active by the addition of normal serum, led Myers to conclude that such a serum is capable of forming precipitoids analogous to toxoids.

The Action of Specific Precipitins upon Egg-Albumin, Blood and Blood Derivatives.

The credit of having first observed the formation of specific precipitins in the blood of animals treated with various sera belongs to Tchistovitch, as stated by Bordet (March, 1899). Tchistovitch (May, 1899) inoculated rabbits with eel-serum, which is toxic, and thereby obtained a specific antitoxic serum from these animals, as also from similarly treated goats, dogs, and guinea-pigs. In addition to the antitoxic property acquired by the serum it was found to acquire the property of producing a precipitate when it was added to eel-serum. When but a small amount of toxin was present the precipitin acted but feebly. Normal sera gave no such reaction. Adopting the terminology of Myers, I shall hereafter refer to the precipitate formed in a serum on the addition of its specific anti-serum as a *precipitum*.

Tchistovitch found that the precipitum was soluble in dilute acids

[1] *Lehrb. d. physiol. Chem.*, Jena, 1893.

and alkalis, but that it was insoluble in water, as also in solutions of alkaline carbonates and neutral salts. The precipitum is only formed in alkaline solutions. In neutral solutions there may be some opalescence, whilst acid solutions remain clear. The precipitum is non-toxic to rabbits by intravenous injection, although the clear fluid may be toxic if it contains unneutralized toxin. It is an interesting fact that the precipitin disappears in animals which have been treated for some time, having been rendered immune. Eel-serum heated to 58° C. gave less precipitum on the addition of its anti-serum. The reaction proved negative with eel-serum which had been heated to about 80°.

Tchistovitch next injected rabbits with horse-serum, the animals receiving 5 to 6 injections of 3 c.c. at a time. The treated rabbits yielded an anti-serum which produced a precipitum with horse-serum, but not with that of the donkey, nor of the normal rabbit. Tchistovitch draws attention to the fact that agglutinins are different bodies to the precipitins, for no precipitins are formed in the serum of animals treated with *B. tetani*, whereas these organisms are agglutinated by antitetanic serum[1].

Bordet (March, 1899) treated rabbits with intraperitoneal injections of defibrinated fowl's blood, and observed that their serum acquired great agglomerating and haemolysing power, and in addition produced a precipitum on being added to normal chicken-blood. As in Tchistovitch's experiments with eel-serum, the precipitum in this case was soluble in dilute alkaline solutions. This was subsequently (15 Nov. 1900) confirmed by Uhlenhuth in so far as he found the serum of rabbits treated by intraperitoneal injections of chicken-blood to contain a precipitin for dilute solutions of chicken-blood. Uhlenhuth states that his anti-serum exerted no effect on the blood of the horse, ox, sheep, and pigeon. Bordet however (p. 233) states that the anti-serum for chicken-blood *does* produce a precipitum with pigeon-blood[2].

Bordet states that when guinea-pigs are treated with rabbit-serum no anti-body is formed. Nolf (May, 1900) has made a similar observation on pigeons treated with chicken-blood. It seems therefore that precipitins are not always formed in the bodies of animals treated with different sera.

Nolf collected about 10 c.c. of chicken-blood by allowing it to flow into 3 to 4 times its volume of 1% salt solution. After separating the

[1] See also Bordet (1899, p. 233) and the results of Myers (1900) quoted below.

[2] See analogous results with experiments on chicken and pigeon egg-albumin to be mentioned presently.

corpuscles from the plasma he injected these separately into two sets of rabbits. The animals received 4 to 6 injections at intervals of 4 to 5 days. Only the serum-treated rabbits yielded a precipitin for chicken-blood, the serum of the others had no more effect on chicken-blood than has normal rabbit-serum. Two other series of rabbits treated respectively with a dog's blood corpuscles and serum gave identical results, specific precipitins being formed in serum-treated rabbits, which had only a precipitating action on dog's blood.

Nolf obtained an anti-serum for rabbit-blood by treating chickens with their serum. He confirmed the observation of Tchistovitch that horse-serum produces an anti-serum when injected into rabbits.

Nolf saturated anti-serum with magnesium sulphate, and purified the globulin precipitate by redissolving it, and afterwards reciprocating it with the same salt. He obtained the albumin by adding 1% acetic acid to the filtrate from the globulin precipitate. He removed the magnesium salts by dialysis during eight days with chloroform-water. He then added 1% NaCl to the neutral fluid, and sterilized it by exposure for half-an-hour to a temperature of 56° C. on eight successive days.

Rabbits treated with globulin-solution yielded a precipitin, whereas those treated with albumin-solution did not. Consequently the precipitum is due to the action of bodies formed in animals as the result of the reaction produced by globulin-injections. The chemical treatment to which Nolf subjected the serum evidently did not materially alter the composition of the globulin, for the artificial globulin-solution produced an anti-serum of the same character as that resulting from treating the animals with normal serum. By adding anti-serum to albumin and globulin-solutions, as also to mixtures of these, Nolf was able to prove that the precipitum is a globulin.

Myers (14 July, 1900) injected proteids (crystallized egg-albumin from the white of the fowl's egg, sheep and bullock serum-globulin, and Witte's peptone) intraperitoneally into rabbits. The crystallized egg-albumin was prepared by the method of Hopkins and Pinkus[1]. Specific precipitins for solutions of egg-albumin were formed in the serum of the rabbits after they had been treated for some months. The precipitum was formed more rapidly at 37° C. than at room-temperature. A slight precipitum was produced in solutions of the egg-albumin of the duck.

[1] *Journ. of Physiol.*, vol. XXIII.

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Specific precipitins also appeared in the serum of rabbits treated with sheep and ox-globulin solutions. The serum of the rabbits treated with sheep-globulin was found to contain two agglutinating bodies, the one acting on sheep-corpuscles, the other on those of the fowl. The precipitin obtained by treatment with sheep-globulin had a slight effect on ox-globulin, and the converse. On the other hand, the serum from rabbits which had been treated with ox-globulin did not agglutinate the corpuscles of either sheep or ox-blood.

The action of the precipitins in the serum of rabbits treated with the egg-albumin and globulins was not appreciably affected by exposure for half-an-hour to a temperature of 56° C. We have already referred above to Myers' experiments with peptone.

Uhlenhuth (15 Nov. 1900) diluted the white of fowls' eggs in normal salt-solution and injected the fluid in quantities up to 100 c.c. intraperitoneally into rabbits. The animals did not suffer from the treatment. After a rabbit had received 5 to 6 eggs in this way, its serum was found to contain a precipitin which acted on 5 to 10% solutions of chicken egg-albumin. The animals which had received the greatest number of eggs gave the most powerful anti-serum; in one case a positive reaction was obtained with a 1 : 100,000 dilution of egg-albumin. The ordinary chemical tests only gave a reaction with dilutions up to 1 : 1000. Normal rabbit-serum never produced this reaction, and the anti-serum added to various commercial albuminous preparations, outside of those derived from the fowl's egg, constantly gave negative results. The anti-serum, heated for one hour to a temperature of 60° C., gave almost as powerful a reaction as the unheated serum. The reaction is not entirely specific, as the pigeon's egg gave the same reactions, though to a lesser degree. Rabbits treated with solutions of pigeon egg-albumin gave a serum which also acted on chicken egg-albumin, and for this reason we may conclude that the albuminous constituents in both species of eggs are closely allied.

Uhlenhuth moreover made the interesting observation that the precipitin appeared in the blood of a rabbit which was *fed* on egg-albumin, the solution being introduced daily by means of a sound. The precipitin only appeared after this mode of feeding had been continued for 24 days.

Leclainche and Vallée (25 Jan. 1901) treated rabbits by intravenous injections of 20 c.c. of albuminous urine, containing 1 to 2 g. of albumin per liter. At times there were symptoms of intoxication, and the first

injections were not infrequently followed by emaciation. After having been treated for three months, and having received a total of 150 to 200 c.c. of urine, the animals were allowed a rest of 15 days before being bled. The serum of these animals, added to an equal volume of the urine used for treatment, produced almost immediately an albuminous precipitum. The washed precipitum gave all the albumin reactions. No precipitum was formed on the addition of the normal sera of the horse, donkey, sheep, and ox. The amount of precipitum does not depend upon the amount of albumin present in the urine, and although they obtained a precipitum with albuminous urine which had been diluted 1 : 5 or 1 : 10, the best results were obtained by mixture of equal volumes of the urine and test-serum. When heated for 2 hours at 58° C. the test-serum was still effective, whilst the albuminous urine, thus treated, showed a much less marked reaction.

The anti-serum was very active for serum-albumin, but almost indifferent towards urine containing much globulin. A positive reaction was obtained with the urine of three cases of interstitial nephritis, but there was no reaction when the test-serum was added to the urine of a case of parenchymatous nephritis which contained much globulin. Albuminous urine from the horse and cow gave no reaction. A positive reaction was obtained with human pleuritic exudation, but not with human blood-serum.

Uhlenhuth (7 Feb. 1901) injected rabbits intraperitoneally with 10 c.c. of defibrinated ox-blood. After 5 to 6 injections, made at intervals of 6 to 8 days, their serum gave the specific reaction. Bloods of different animals were diluted 1 : 100 with tap-water, the remains of the stroma were removed by sedimentation or filtration, and about 2 c.c. of the clear solution were placed in small tubes about 6 mm. wide. An equal volume of double normal salt-solution (1·6 %) was then added to the blood-solution, this being essential as normal rabbit-serum produces clouding of the watery blood-solution, and this may mask the result. Uhlenhuth used clear solutions of human blood and that of the ox, horse, donkey, pig, sheep, dog, cat, deer, fallow-deer, hare, guinea-pig, rat, mouse, rabbit, chicken, goose, turkey, and pigeon. On adding 6 to 8 drops of ox-blood anti-serum to the various blood-solutions he only obtained a positive reaction with ox-blood.

Similar results were obtained by treating rabbits with human blood, the test-serum in this case only giving a reaction with human blood. He moreover made the important observation that human, horse, and ox-blood which had been dried for 4 weeks on a board, could be readily

distinguished by the test-serum, added to these bloods in normal salt-solution.

As Wassermann and Schütze (18 Feb. 1901) point out, the reactions produced by specific haemolysins and agglutinins are of but little value forensically, for to obtain these reactions a large number of *intact* blood-corpuscles must remain in suspension.

They confirmed the observations of previous investigators in that they obtained anti-sera from animals treated with human and other bloods. They treated the animals by injecting 10 c.c. of the particular blood-serum into rabbits, the injections being made sub-cutaneously about every two days. After the rabbits had received 5 to 6 injections a period of six days was allowed to elapse, after which they were bled to death, and the blood was placed in the ice-chest. The precipitum was formed more rapidly at 37° than at room-temperature.

Wassermann and Schütze tested their anti-sera on 23 kinds of blood: that of the horse, donkey, goat, cow, ox, sheep, pig, dog, cat, baboon, guinea-pig, rabbit, house-mouse, house-rat, goose, duck, chicken, sparrow, eel, pike, and tench. On adding the test-serum obtained from rabbits treated with human blood, none of the bloods reacted except human and baboon blood. The blood of the baboon, however, reacted much more slowly and incompletely to the test, than did the human blood.

Tests were moreover made with blood which had been allowed to dry on knives, linen, etc. The blood which had been dried for 3 months, was dissolved in normal salt-solution, and filtered, so as to obtain a clear solution. About 5 to 6 c.c. of salt-solution were added to a dried drop about the size of a sixpence, and about 0·5 c.c. of the test-serum were added to the blood-solution. The reaction was well marked after 20 minutes at 37° C. They state that the reaction was more powerful with fresh anti-serum than with serum which had been preserved for some time; nevertheless, a positive reaction was obtained with a test-serum which had been kept for two weeks on ice.

Stern (28 Feb. 1901) reports similar experiments. He injected rabbits every two or more days, according to the health of the animal, the dose of serum being 5 to 10 c.c. After 2 to 3 weeks the serum of the rabbits contained a precipitin, which only acted on human blood, whether fluid, or dried and made into solution, and on albuminous urine. The result was negative when the blood-serum of the horse, ox, sheep, and pig were tested. He also found the reaction feeble though

positive with the blood of three species of monkeys, a species of *Cercopithecus* ("Meerkatze"), *Macacus cynomolgus* L. ("Java-Affe"), and the "Kronen-Affe." According to Stern the reaction is therefore not strictly specific.

By long-continued treatment the amount of precipitin can be greatly increased. Stern states that he has a rabbit the serum of which gives a positive reaction when added to human blood diluted 1 : 50000.

Mertens (14 March, 1901) treated rabbits by intraperitoneal injections of egg-albumin of the fowl, as also subcutaneous and intravenous injections of human blood-serum, his results confirming those of the previous observers. He also treated rabbits with albuminous urine, but found that he obtained a greater amount of precipitum when he used the serum-treated animals' blood, instead of that from rabbits which had been injected with albuminous urine. This was doubtless due to the greater amount of the anti-body produced by serum-injections. He concludes that the albumin in urine must be derived from the blood, for otherwise it would not be acted on by the precipitins in the blood of serum-treated animals. He made the interesting discovery that the blood of a young rabbit, born of a serum-treated mother, gave the typical reaction.

Dieudonné (2 April, 1901) treated rabbits by means of human albuminous urine, pleural exudation, and blood-serum. He injected quantities of 10 c.c. every 3 to 4 days. He made his tests according to Uhlenhuth's method, and found that he obtained no reaction with four other bloods, i.e. of the rabbit, guinea-pig, pigeon, and goose. Though the animals had been treated in various ways, they all gave practically the same test-serum. Normal rabbit-serum had no effect on human blood-solutions.

We come finally to the observations of Zuelzer (4 April, 1901). He made the same experiments as Mertens. He injected 5 to 10 c.c. of albuminous urine into rabbits every day to every third day, the rabbits yielding a precipitin at the end of 2 to 3 weeks. The urine he injected contained 1 to 9 $^0/_{00}$ albumin. Though this proves that one albuminous body in the tested urine was derived from the blood, he does not consider that it justifies Mertens' generalization that all albuminous bodies in nephritic urine are derived from the blood.

Methods.

In the following experiments only rabbits were used for obtaining the specific anti-sera. The blood, pleuritic exudation, etc., was injected intraperitoneally in quantities of 5 and 10 c.c., usually beginning with the smaller dose, the amount of 10 c.c. not being surpassed. Only a few injections were made subcutaneously, intraperitoneal injection being preferred. The animals were so little affected by the operation that the males frequently sought to copulate with the females immediately after being released from the hands of the assistant, who held them belly upwards on a high stool. The abdomen was shaved over the seat of operation, and the skin disinfected with lysol. The skin was punctured by means of a small scalpel in the lower left-hand region of the abdomen. The somewhat blunted hypodermic needle was then introduced through the puncture and gently bored through the abdominal wall, through which it passed with a jerk. After the injection had been made, and the needle withdrawn, the parts were dried with sterilized cotton, there being no bleeding, and tincture of benzoin was applied over the small slit (usually 1·5 to 2 mm. long) in the skin. The syringe used was entirely made of metal, the piston being lubricated with sterilized vaseline. It was disinfected before use by means of lysol solution, which was also used for rinsing it out, when several injections were made in succession with the various sera, and into different animals. The animals were tattooed upon the inner side of the ear with a letter and number, the letters *D*, *H*, etc. indicating the kind of blood injected. This excluded any possible confusion. The animals were regularly weighed every day, or every second day, and the injections were not repeated until they had regained any lost weight. The loss in weight was generally small and usually less after the first two injections.

We only lost three animals; in one, a young animal, death was due to Psorospermiasis; in the second the cause of death could not be determined at the autopsy. The third animal (ox-serum-treated rabbit No. II, see Protocol) died of an inter-current disease. There was no peritonitis or bacterial infection in any of these cases. We never observed any symptoms of intoxication consequent upon the serum-injections, the sera used being those of the dog, ox, sheep, horse, cat, and man, besides human pleuritic exudation.

Effective anti-sera were obtained after the fifth or sixth injection,

sometimes earlier, as will be seen from the Protocols. The animals were periodically bled by puncturing the lateral ear-vein, the skin having been shaved, disinfected with lysol, and dried with sterilized cotton. The blood, as it flowed from the vein, was collected in fine-pointed sterilized bulbed pipettes from which it was expelled into test-tubes which were laid almost horizontally into racks.

We obtained a considerable quantity of serum in this way from small amounts of blood, the tubes being placed vertically in test-tube racks after the coagulum had formed. Ten to forty c.c. of blood were readily obtained from the ear-vein by this method. In other cases the rabbits were bled to death by cutting the carotids and catching the blood in large flat dishes and Petri-dishes, these being subsequently tilted and the serum placed in corked bottles to which chloroform was added. This method served the purpose practically as well as when we pipetted off the sterile serum from the test-tubes and sealed it in glass tubes. We have found that neither drying for some weeks, nor the addition of chloroform to anti-sera and normal sera, prevents the reaction taking place.

Normal serum was sometimes sterilized by filtration, and preserved in sealed tubes. At other times it was dried and solutions of the dried substance were injected. Sera which had been collected with ordinary precautions as to cleanliness, and to which lysol or chloroform had been added, were also used. In the last case the serum that was to be injected, was poured into sterile Petri-dishes which were placed uncovered in the thermostat until the chloroform had evaporated.

We have found it very convenient to preserve normal serum and defibrinated blood on strips of filter-paper, which we have immersed in blood and hung up to dry, pinning them to the edge of a table or bench. It is best to keep one end of the strip clean, so as to make pencil-notes upon it. It is possible to roughly estimate the amount of blood or serum absorbed by these strips, and to cut out squares of suitable size for purposes of testing. Whereas the blood and serum which is dried on horizontal glass plates in the incubator-room only dissolves slowly and frequently gives clouded solutions, the filter-paper rapidly gives off the soluble ingredients of the blood in perfectly clear solution, the fibres of the permeable paper retaining the fine particulate matter and allowing the solvent to act rapidly. We have also preserved some of our anti-sera in this way.

In the following protocols the weights of the animals are given thus, "2500—2490 g.," this signifying that the weight at the beginning of the

experiment was 2500 g., and at the conclusion 2490 g. The test for specific precipitins was made by adding about 3 drops of the treated animal's serum to a clear filtered 1 : 100 dilution of the blood, etc. with which it had been treated. Fluid serum was diluted with normal salt-solution. Dried serum was dissolved in ten parts of normal salt-solution and this diluted 1 : 100. Dry blood was dissolved by means of water, as recommended by Uhlenhuth, to which an equal quantity of double NaCl solution (1·6 %) was subsequently added. The results of the tests are given in the following protocols of our experiments. Where much precipitin was formed we refer to the reaction as "marked," etc. The test-tubes used contained about 0·5 c.c. of blood-dilution. When test-serum flowed to the bottom of the tube the reaction was most striking at the line of contact between the fluids.

I. *Rabbits treated with Dog-Serum.*

I. Weight 2460—2410 g. Treatment lasted 51 days. Received 6 injections, the first of 5 c.c. subcutaneously, the rest of 10 c.c. intraperitoneally, the last injection being made one week before the animal was bled to death from the carotids.

Bled 20 c.c. from ear after injection 3. *Marked reaction.*
„ 20 c.c. „ „ „ 5. „ „

II. Weight 1720—1850 g. Under treatment 50 days. Received 7 injections, the first and fifth of 8 c.c., the rest of 10 c.c. intraperitoneally.

Bled 10 c.c. from ear after injection 3. *Reaction.*
„ 15 c.c. „ „ „ 4. *Marked reaction.*

The serum obtained from the first bleeding contained less precipitin than that from the second.

III. Weight 2300—2170 g. Under treatment 51 days. Received 6 injections, the first and third of 6 c.c., the rest of 10 c.c., intraperitoneally. Bled to death from carotids seven days after last injection.

Bled 15 c.c. from ear after injection 3. *Reaction.*
„ a few c.c. „ „ „ 5. *Marked reaction.*

The reaction obtained with the serum obtained from the second bleeding was much greater than at the first bleeding.

II. *Rabbits treated with Sheep-Serum and Defibrinated Blood.*

I. Weight 2270—2030 g. Under treatment 66 days. Received 7 intraperitoneal injections, the first of 5 c.c. defibrinated blood, the second of 5 c.c. fluid serum, the third of 7 c.c. normal solution of dried serum, the rest of 10 c.c. filtered serum.

Bled 25 c.c. from ear after injection 4. *Very slight reaction.*
„ 4 c.c. „ „ „ 7. *Marked reaction.*

II. Weight 2220—2970 g. Under treatment 66 days. Received 6 injections, the first intraperitoneally of 5 c.c. defibrinated blood, the second subcutaneously of 7 c.c. filtered serum, the third of 10 c.c. normal solution of serum, the rest of 10 c.c. filtered serum intraperitoneally. Bled to death from carotids.

Bled 15 c.c. from ear after injection 4. *Marked reaction.*
„ 15 c.c. „ „ „ 5. „ „

There was no appreciable difference between the reactions produced by the serum from these two bleedings, when added to a 1 : 100 dilution of sheep's blood.

III. *Rabbits treated with Ox-Serum.*

I. Weight 1970—1700 g. Under treatment 66 days. Received 9 injections, the first of 5 c.c. intraperitoneally, the second of 7·5 c.c. subcutaneously, the rest of 10 c.c. intraperitoneally. Injections 5 and 6 were made with normal solution of dried serum, the last three with serum preserved with chloroform.

Bled a few c.c. from ear after injection 5. *Marked reaction.*
„ 25 c.c. „ „ „ 8. „ „

The serum used for testing in the last case had been preserved 8 days in a sealed tube at room-temperature. It was added to a 1 : 100 dilution which had been left standing for 13 days with chloroform. There was no marked difference in the reaction produced by the first and second serum.

II. Weight 2220—1810 g. Under treatment 55 days, its weight having fallen from 1980 g. during the last 6 days. The animal was killed by being bled from the carotids. At autopsy the left kidney was found to be hydronephrotic, the spleen pale, the retroperitoneal glands much enlarged and caseous. The bacteriological examination was negative.

Bled 35 c.c. from ear after injection 4. *Marked reaction.*
„ 10 c.c. „ „ „ „ 7. „ „

The reaction obtained with the second serum was somewhat more marked.

III. Weight 2500—2490 g. Under treatment 66 days. Received 7 intraperitoneal injections, the first of 5 c.c., the second of 7·5 c.c. (in part subcutaneously), the fourth of 7 c.c., the rest of 10 c.c. Injection 5 was made with normal solution of serum, the last two with serum preserved with chloroform.

Bled 10 c.c. from ear after injection 5. *Marked reaction.*

IV. *Rabbits treated with Horse-Serum.*

I. Weight 1370—1700 g. Under treatment 30 days. Received 6 intraperitoneal injections, the first of 5 c.c., the rest of 10 c.c. This animal was treated entirely with an old weakly antitoxic serum for diphtheria, preserved with 0·4% trikresol in a corked bottle for 2 years and 7 months.

Bled 6 c.c. from ear after injection 6. *Reaction.*

II. Weight 2180—2170 g. Under treatment for 30 days. Received 5 intraperitoneal injections, the first of 5 c.c., the rest of 10 c.c. The first two injections were made with the old trikresol serum mentioned under Rabbit I, the rest with filtered horse-serum preserved with chloroform.

Bled 20 c.c. from ear after injection 5. *Marked reaction.*

III. Weight 1970—2150 g. Under treatment for 30 days. Received the same treatment as the preceding rabbit.

Bled 15 c.c. from ear after injection 5. *Reaction.*

V. *Rabbits treated with Cat-Serum.*

Both of these animals were treated with normal cat-serum, collected aseptically and preserved in sealed tubes until used.

I. Weight 1620—1950 g. Under treatment 58 days. Received 9 intraperitoneal injections, the first, fourth and fifth of 4, 8 and 9 c.c. respectively, the rest of 10 c.c.

Bled about 50 c.c. from ear after injection 5. *No reaction.*
„ 5 c.c. „ „ „ „ 9. „

II. Weight 1720—2370 g. Under treatment 58 days. Received 8 intraperitoneal injections, the first of 4 c.c., the second of 6 c.c., the rest of 10 c.c.

Bled 10 c.c. from ear after injection 4. *No reaction.*
„ 5 c.c. „ „ „ „ 8. „

VI. *Rabbits treated with Human Blood and Pleuritic Exudation.*

I. Weight 3070—2930 g. Under treatment 46 days. Received 6 intraperitoneal injections, each of 10 c.c. With the exception of injection 5, which was made with fresh serum, the animal only received pleuritic exudation which had been preserved for 5 to 6 months by the addition of chloroform, having been kept in a corked bottle at room-temperature.

Bled 30 c.c. from ear after injection 4. *Reaction slight.*
„ 40 c.c. „ „ „ 5. *Marked reaction.*

II. Weight 3120—3040 g. Under treatment 46 days. Received 6 intraperitoneal injections, each of 10 c.c., the third and fourth with filtered serum, the rest with the old pleuritic exudate, mentioned under Rabbit I.

Bled 20 c.c. from ear after injection 4. *Reaction.*
„ 40 c.c. „ „ „ 5. *Marked reaction.*

III. Weight 1690—1620 g. Under treatment 39 days. Received 5 intraperitoneal injections, the second of 7 c.c., the rest of 10 c.c. of filtered serum, fresh pleuritic exudation, and exudation to which chloroform had been added.

Bled 4 c.c. from ear after injection 5. *Reaction.*

IV. Weight 1524—1680 g. Under treatment 39 days. Received 6 intraperitoneal injections, the first of 6 c.c., the rest of 10 c.c. as in Rabbit III.

Bled 8 c.c. from ear after injection 3. *No reaction.*

The protocols of our experiments show that we obtained precipitins in the blood of rabbits treated with dog, sheep, ox, horse, and human blood. Our results have been negative in rabbits treated with the blood of the cat. The analogous observations of Bordet and Nolf have already been referred to on p. 371.

The rest of the animals yielded an anti-serum which produced a

marked reaction (much precipitum) after the third injection, in some the reaction only took place after the fifth injection. This does not seem to depend upon differences in the weights of the animals in relation to the dose of serum, but upon individual differences in the reacting power of the rabbits.

We have tested 36 kinds of blood up to the present[1], the bloods used being those of man, four species of monkey: *Cercopithecus campbelli* Waterh., *Cercopithecus patas*, W. coast of Africa, *Cercopithecus lalandii* Is. Geoffr. [S. Africa], *Macacus rhesus* [India], the Rufous Rat-Kangaroo (*Hypsiprymnus rufescens* [Gray], N. S. Wales), the Capybara (*Hydrochoerus capybara*, S. America), the polecat (*Mustela putorius*), Suricate (*Suricata tetradactyla* [S. Africa]), squirrel (*Sciurus vulgaris*), guinea-pig, tame and wild rabbit (*Lepus cuniculus*), white rat, black rat (*Mus rattus*), horse, ox, sheep, white-tailed gnu (*Connochaetes gnu*, S. Africa), gazelle (*Gazella arabica*), deer (*Cervus axis* Erxl.; India), dog, cat, pig, bat (*Plecotus auritus*), pigeon, chicken, pheasant, swan (*Cygnus olor*), duck, chaffinch (*Fringilla coelebs*), cross-bill (*Nucifraga caryocatactes*), rook (*Corvus fragilegus*), swallow (*Hirundo urbica*), corn-crake (*Crex pratensis*), frog (*Rana temporaria*), newt (*Molge cristata*), snake (*Tropidonotus natrix*)[2].

The serum of rabbits treated with *dog-serum*, added to all these bloods, gave a negative reaction throughout, excepting in the case of the dog. The tested dog-blood was dried and dissolved in salt-solution, or used in the form of diluted fluid serum. Whereas a marked and almost immediate precipitation occurred on the addition of the specific anti-serum to dog's blood, all the other blood-solutions remained perfectly clear.

The serum of rabbits treated with *sheep-serum* produced a marked precipitum with sheep-serum or blood-solution, as also a distinct but less marked reaction with the blood of the gazelle and axis deer. All the other sera and bloods remained perfectly clear, excepting those of the ox, squirrel and swan, in which there was very slight clouding.

The serum of rabbits treated with *ox-serum* only produced a marked precipitation in ox-serum dilutions, or dried ox-blood solutions. A distinct reaction was obtained with the blood of the gazelle and axis deer. All the other bloods gave a negative reaction, a slight clouding only being produced in blood-solutions of the sheep, gnu, squirrel and swan.

[1] The investigations on different bloods are being pursued and will be reported later.

[2] We are indebted to Frank E. Beddard, Esq., F.R.S., Prosector of the Zoological Society's Gardens, London, for nine of these bloods.

The serum of rabbits treated with *horse-serum* only produced precipitation in dilutions of horse's blood or serum, not even a clouding in any of the other bloods noted.

The serum of the rabbits treated with *human* blood, serum, and pleuritic exudation only produced a marked precipitation in human blood-solutions, etc. The blood of the four monkeys gave a slight but distinct reaction. A very faint clouding appeared in the solutions of the bloods of the horse, ox and sheep, whereas all the other bloods remained perfectly clear. The test gave positive results when made with diluted human serum, pleuritic exudation, both fresh and putrid, blood and serum which had been dried on filter-paper and on glass plates, with blood which had undergone putrefaction for two months, with the blood of several persons who had cut themselves (blood collected on filter-paper), with the serum from a blister on the foot following upon a long walk, and with the serum from a blister following a burn on the hand. Both nasal and lachrymal secretion gave a slight but decided reaction. A faint clouding was produced in normal urine. That the precipitum formed in putrid blood dilution was specific was proved by adding the anti-sera of rabbits treated with ox, sheep and dog-serum to the blood dilution, no reaction resulting.

The tests made with dried blood, whether dried on glass or filter-paper, gave us perfect reactions, as did also 1:100 dilutions kept for two weeks in test-tubes in the laboratory. Although chloroform had been dropped into the bottom of these tubes, moulds occasionally developed upon the surface of the serum, but this seemed in no way to interfere with the specific reaction. Strips of filter-paper upon which both sheep and ox-blood had been allowed to dry were placed under different conditions. Some were kept for 2 months at 37° C., in the dark; others at room-temperature in the dark, and in diffused light for the same period; others again were exposed for eight days to the action of sunlight in a window. All of these samples gave apparently just as good reactions as fresh bloods, though of course our method cannot as yet be strictly considered to be quantitative. Both the body in the serum which is acted upon by the anti-serum as also the specific body in anti-serum seem to be about equally resistant. Anti-serum dried for 42 days on filter-paper and then dissolved in salt-solution was found to give a perfectly characteristic reaction when added to its homologous (ox) serum, the latter diluted (1:100) as usual; it did not however produce a reaction in dilutions of other bloods. Dried normal sera exposed for half-an-hour to a temperature of 100° C. still gave a clear reaction, as did also 1:100 dilutions exposed for half-an-hour to 55°.

As I first showed the bactericidal properties of blood are destroyed at the latter temperature (see footnote, p. 367). Dilutions of blood exposed to a temperature of 100° gave no reaction.

The first rabbit in the series treated by horse-serum injections received old antitoxic serum which had been kept at room-temperature in the laboratory for *two years and seven months.* We are indebted to Dr Louis Cobbett for this serum. The serum, to which trikresol had been added, had been kept in a corked bottle, exposed to diffused light, the temperature of the room being very high during the summer months. The first and second rabbits of the series treated with human pleuritic exudation, etc., received only one and two injections respectively of fresh serum, being treated for the rest of the time with pleuritic effusion which had been kept at room-temperature for *five to six months.* The pleuritic fluid had been preserved in a corked bottle, with chloroform. These observations seem to me to possess a particular interest.

It seemed of interest, from a medico-legal standpoint, to determine whether or no a *mixture* of two kinds of blood would prevent the detection of one of the bloods in the mixture, the presence of another blood might inhibit the action of the anti-serum. To determine this question 1 : 100 dilutions of two kinds of blood were mixed together in equal proportions and tested as shown in the following table.

Table showing the results of tests made with blood-mixtures.

Bloods mixed	Anti-serum used	Result	No reaction produced by non-homologous anti-sera or normal rabbit-serum
Human and ox	Human*	Marked reaction	Horse*
,, ,, sheep	,,	,, ,,	Dog
,, ,, dog	,,	,, ,,	Ox
,, ,, horse	,,	,, ,,	Sheep
,, ,, cat	,,	,, ,,	Dog
Ox and human	Ox	,, ,,	Normal rabbit serum
,, ,, dog	,,	,, ,,	,, ,,
,, ,, horse	,,	,, ,,	,, ,,
Dog and human	Dog	,, ,,	,, ,,
,, ,, horse	,,	,, ,,	,, ,,
,, ,, ox	,,	,, ,,	,, ,,
Sheep and human	Sheep	,, ,,	,, ,,
,, ,, dog	,,	,, ,,	,, ,,
,, ,, horse	,,	,, ,,	,, ,,
Horse and human	Horse	,, ,,	,, ,,

* The names in this column indicate the blood with which the rabbit was treated which yielded the anti-serum.

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Each blood in the mixtures included in the table was actually diluted to 1 : 200 by the addition of the equal volume of other blood dilution.

From a medico-legal standpoint, it seemed important to determine whether the reaction would take place in the presence of several other bloods. For this reason three different mixtures were made, as follows:

Mixture I. contained equal volumes of ox, sheep, horse, cat, and human blood dilutions (each about 1 : 100). When the anti-serum for human blood was added to this mixture, a marked reaction immediately took place, whereas no reaction followed when we added normal rabbit or cat serum, as also anti-serum for dog's blood.

Mixture II. contained equal volumes of human, dog, horse, sheep, ox, and newt blood dilutions (each about 1 : 100). Reactions were obtained on the addition of the anti-sera for human and dog blood, whereas both normal rabbit serum and cat serum produced no reaction.

Mixture III. contained equal volumes of dog, gazelle, swallow, wild rabbit, duck and snake blood dilutions (each about 1 : 100). A marked reaction was obtained on the addition of the anti-serum for dog's blood, whereas no reaction followed upon the addition of the anti-serum for human blood, as also upon the addition of cat serum, and the serum of a normal rabbit.

We see from these experiments that a blood can be detected even when mixed with that of several other animals, each blood in the mixture being diluted about 1 : 500 or 1 : 600.

CONCLUSIONS.

1. The investigations we have made confirm and extend the observations of others with regard to the formation of specific precipitins in the blood-serum of animals treated with various sera.

2. These precipitins are specific, although they may produce a slight reaction with the sera of allied animals.

3. The substance in serum which brings about the formation of a precipitin, as also the precipitin itself, are remarkably stable bodies.

4. The new test can be successfully applied to a blood which has been mixed with that of several other animals.

5. We have in this test the most delicate means hitherto discovered of detecting and differentiating bloods, and consequently we may hope that it will be put to forensic use.

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