Spleen.—Weight 14 ozs. Feels hard to the knife on section. Pale appearance and excess of fibrous tissue on cutting open.

Portion preserved for microscopic examination in England.

Kidneys.—Right, weight 5 ozs. Left, 6 ozs. Both were swollen, the kidney substance bulging from the capsule when cut into.

Both capsules were a little adherent.

Cortices undiminished. The whole kidney substance appeared a little paler than normal.

Portions preserved for microscopic examination in England.

Pancreas and Suprarenals.—Normal.

Stomach.—Slightly dilated. No gastritis. Some *post-mortem* staining.

Intestines.—Normal. Mesenteric and retroperitoneal glands not enlarged.

Brain.—Calvaria normal. Weight of brain, 52 ozs. No thickening of membranes. Brain substance normal. Ventricles normal in size and no excess of fluid.

Portions of cerebral cortex and cerebellum preserved for examination in England.

(Signed) H. B. Owen,
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Auto-Agglutination of Red Blood Cells in Trypanosomiasis.

By Warrington Yorke, M.D.

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(From the Runcorn Research Laboratories of the Liverpool School of Tropical Medicine.)

CONTENTS.

Introduction.—It is now a well recognised fact that the erythrocytes in fresh preparations of the blood of Sleeping Sickness cases and animals

infected with trypanosomiasis frequently exhibit a more or less marked degree of agglutination. Attention was first drawn to this phenomenon n 1898 by Kanthack, Durham and Blandford,* who found that the red blood cells of animals infected with nagana, instead of forming rouleaux, tended to clump together into masses and to lose their outlines.

More recently Christy† (1904), Dutton and Todd‡ (1905), Martin, Lebœuf and Roubaud§ (1906-8), and others have described a similar condition in fresh preparations of the blood of patients suffering from Sleeping Sickness.

Dutton and Todd,‖ referring to this condition of the blood, wrote: "Only once have we had the opportunity of observing a patient (European) from whose blood trypanosomes, once present, have finally disappeared. In this instance auto-agglutination of the red cells disappeared with the parasites.

Later, it has been noted by many investigators¶ that the phenomenon gradually disappears in animals after the successful drug treatment of the disease.

In spite of the fact that auto-agglutination of the red cells in trypanosomal infections has attracted so much attention, very little work appears to have been done with a view to determining the nature of the changes in the blood which lead to its manifestation. Most of the workers have contented themselves with simply recording the presence of the phenomenon. So far as have been able to ascertain from a search of the literature, only two authors mention any details regarding the mechanism of its production.

Kanthack, Durham and Blandford stated that the serum of the blood of animals exhibiting auto-agglutination, when added to normal blood o the same species of animal, caused the red corpuscles to clump together. On the other hand, at a recent meeting of the Society of Tropical Medicine and Hygiene, in the discussion following Dr. Bagshawe's paper on "Recent

* "On Nagana or Tsetse Fly Disease," 'Roy. Soc. Proc.,' 1898, vol. 64, p. 100.

† "Sleeping Sickness," 'British Medical Journal,' 1904, p. 1456.

‡ "Gland Puncture in Trypanosomiasis," 'Memoir of the Liverpool School of Tropical Medicine,' 1905, No. 16, p. 99.

§ 'Rapport de la Mission d'Études de la Maladie du Sommeil au Congo Français,' 1906—8, p. 281.

‖ *Loc. cit.*

¶ Thomas and Breinl, "Pathology and Treatment of Trypanosomiasis," 'Memoir of the Liverpool School of Tropical Medicine,' 1905, No. 16.

Mensil, Nicolle, and Aubert, "Recherches sur le Traitement des Infections Expérimentales à *T. gambiense*," 'Annales de l'Institut Pasteur,' 1907, vol. 21, p. 1.

Laveran and Thiroux, "Sur le Traitement des Trypanosomiases," 'Bulletin Soc. Path. Exot.,' 1908, p. 28.

Martin and Darré, "Sur les Symptômes Nerveux du Début de la Maladie du Sommeil," 'Bulletin Soc. Path. Exot.,' 1908, p. 15.

Advances in our Knowledge of Sleeping Sickness," Breinl* remarked that with regard to its (auto-agglutination) mechanism he had not, up to the present, been able to arrive at any definite conclusions. All his attempts to isolate a hæmagglutinin had failed, and he had not been able to demonstrate either an iso- or an auto-agglutinin. Dr. Nierenstein† had shown that in trypanosomiasis a definite increase of the acidity of the blood occurred. This was most probably due to an increase of the amino-acids in the blood, and Breinl and Nierenstein inclined to the view that auto-agglutination was caused by this excess of amino-acids. Unfortunately, they had not so far been able to find a definite proof of the correctness of this conception.

The results obtained by these investigators are thus somewhat conflicting, for whereas Kanthack, Durham, and Blandford found an iso-agglutinin in the serum of certain animals infected with *T. brucei*, Breinl was unable to demonstrate the existence of either an auto- or an iso-agglutinin.

Examination of Serum of Infected Animals for Auto-agglutinin.—Having under observation a number of animals, the blood of which when examined in cover-slip preparations exhibited the phenomenon of auto-agglutination to a greater or less degree, it was decided to perform experiments with a view to investigating the mechanism of its production.

Technique.—The blood was collected in a solution containing 1 per cent. sodium citrate and 0·9 per cent. sodium chloride in distilled water. The blood was then centrifugalised and the corpuscles washed three times in normal saline solution. A 5-per-cent. suspension of the washed red cells was then made in normal saline. Another sample of the same blood was collected and allowed to clot and the serum subsequently freed from the clot by centrifugalisation.

Equal amounts of the serum and emulsion of red corpuscles were then mixed together and drawn up into a capillary tube which was placed vertically in the incubator at 37° C.

The results of such experiments may be briefly summarised. With the exception of a few cases in which there appeared to be a trace of agglutination they were all negative. Similar negative results were obtained when the sera were examined for the presence of iso-agglutinins and also when the serum was replaced by citrated plasma.

At first one was somewhat at a loss to account for these apparently conflicting results, as even the sera of animals where the cover-slip preparations

* 'Trans. Soc. Trop. Med. and Hygiene,' 1909, p. 29.

† "Observations on the Acidity and Alkalinity of the Blood in Trypanosome Infections," 'Annals of Tropical Medicine and Parasitology,' 1908, p. 227.

exhibited the most marked auto-agglutination of the red cells gave invariably negative results when examined for auto- and iso-agglutinins by this method.

Later it was observed that when the blood from one of the infected animals was allowed to flow from a vein of the ear into a watch-glass containing a small amount of citrated saline solution, the red cells quickly sank to the bottom in little clumps, producing in a marked degree the sandy appearance described by Dutton and Todd.* When, however, the watch-glass and salt solution were warmed to 37° C. and the blood dropped in as previously, this appearance did not result; the red cells remained suspended for a considerable time and only subsided gradually, as in the case of normal blood. So long as the temperature of the watch-glass and salt solution was kept at 37° C., no agglutination resulted, but as soon as the temperature was allowed to fall to about 18° C., the red cells ran together into clumps and the typical sandy appearance was obtained.

These observations served to indicate that temperature played an important *rôle* in the development of the phenomenon. Accordingly, the previous experiment was repeated, but on this occasion three sets of tests were made: the first were placed in the incubator at 37° C., the second were left at laboratory temperature (18° to 21° C.), the last were kept in the ice chest at 0° C. Even at the end of five minutes a certain degree of agglutination of the red cells was noticeable in some of the tubes which had been placed in the ice chest, whilst in 15 minutes the red cells in most of them were completely agglutinated, numerous clumps of various sizes being visible in the clear serum. The reaction was also distinct in many of the tubes kept at the laboratory temperature, but it was neither so marked, nor did it occur so quickly as in those subjected to the lower temperature. As before, no agglutination—or only occasionally a trace—was observable in the tests which had been placed in the incubator at 37° C.

A large number of similar experiments were subsequently performed with the blood of monkeys, donkeys, goats, dogs, rabbits, guinea-pigs, and rats infected with various strains of trypanosomes. As a rule, it was found that a marked degree of agglutination only resulted when the temperature of the mixture of serum and red cells was lowered. Very exceptionally slight traces of agglutination were also seen in the tests carried out at 37° C., but these could not be compared with the intensity of the reaction at low temperatures.

Quite frequently a well-marked auto-agglutination was found to occur, at 0° C., in the control tests made with the blood of normal animals. I shall return to this important point later.

* *Loc. cit.*

Iso-agglutination.—A series of experiments were undertaken with a view to ascertaining whether the sera of those infected animals which possessed the property of agglutinating their own red cells were also capable of producing agglutination of the erythrocytes of other animals of the same species. In every case where auto-agglutination was present, iso-agglutination was found to occur when the serum of the infected animal was added to the red cells of another animal (either normal or infected) of the same species.

Analogous results were obtained with the blood of a case of human trypanosomiasis in Major Ross's clinic in Liverpool. Cover-slip preparations of the blood of this case exhibited a certain degree of auto-agglutination during the six months he was under observation. The following table gives the results of an examination of his blood, drawn 10 days before his death, for auto- and iso-agglutinins.

Table I.—Examination of Sleeping Sickness Serum for Auto- and Iso-agglutinin.

Equal volumes of serum and red blood cell suspension used.		Temp.	Result.
5 per cent. suspension of washed erythrocytes in normal saline solution.	Source from which serum was obtained.		
		° C.	
Sleeping Sickness.....	Sleeping Sickness:.....	37	No agglutination in 30 mins.
		18	Marked „ 30 „
		0	Complete „ 10 „
Normal individual A ...	Sleeping Sickness.....	37	No agglutination in 30 „
		18	Complete „ 30 „
		0	„ „ 10 „
Normal individual B ...	Sleeping Sickness.....	37	No agglutination in 30 „
		18	Marked „ 30 „
		0	Complete „ 10 „
Normal individual C ...	Sleeping Sickness.....	37	No agglutination in 30 „
		18	Marked „ 30 „
		0	Complete „ 10 „
Normal individual C ...	Normal individual C	37	No agglutination in 30 „
		18	„ „ 30 „
		0	„ „ 30 „
Normal individual C ...	Normal individual B	37	No agglutination in 30 „
		18	„ „ 30 „
		0	Marked „ 30 „

Spontaneous Agglutination of the Red Cell Suspensions.—On rare occasions it was found that the 5-per-cent. suspension of red cells which had been washed

three times in large volumes of 0·9-per-cent. sodium chloride solution underwent a spontaneous agglutination in the entire absence of serum. Indeed, in one or two instances, where the animals exhibited an extreme degree of auto-agglutination, some difficulty was experienced in obtaining an even suspension of the erythrocytes. The probable explanation of this spontaneous agglutination is that it was due to the absorption of agglutinin from the plasma by the red cells immediately after the blood was shed into the cold citrated saline solution.

This difficulty was obviated by collecting the blood in warm citrate solution and then rapidly centrifugalising and decanting off the citrated plasma. The red cells were then washed thrice in warm normal saline solution. Suspensions prepared in this way exhibited no tendency to spontaneous clumping.

It might be mentioned in this connection that Klein* has succeeded in obtaining agglutinating solutions by grinding up with quartz sand the well-washed erythrocytes of certain animals (rabbit, dog, hen, and guinea-pig). These extracts sometimes agglutinated the red cells of other animals, and frequently also the erythrocytes of the same kind of animal, and even those of the same animal.

Absorption of Agglutinin by Red Cells.—Experiment. To one volume of the citrated plasma of Rabbit 896 (infected with *T. dimorphon*), which caused great agglutination when added to its own red cells and to those of normal rabbits, were added five volumes of the undiluted well-washed red cells of the same animal. The mixture was then divided into two equal portions, A and B. A was placed in the incubator at 37° C. and B in the ice chest at 0° C. At the end of three hours the extracted plasmas were separated from the red cells by centrifugalisation, and were examined for auto- and iso-agglutinins.

Table II.

Equal volumes of extracted plasma and erythrocyte suspension used. Temp. of experiment 0° C.		Result.
5 per cent. suspension of washed erythrocytes in normal saline solution.	Extracted plasma.	
Rabbit 896	A	Complete agglutination in 10 mins.
„	B	No agglutination in 60 mins.
Normal rabbit	A	Complete agglutination in 10 mins.
„	B	Slight agglutination in 60 mins.

* "Beiträge zur Kenntniss der Agglutination rother Blutkörperchen," 'Wien. Klin. Woch.,' 1902, No. 16, p. 413.

The plasma which had been in contact with the red cells at 0° C. had almost completely lost its agglutinating action, whilst the other portion, B, which had been placed in the incubator, had retained its auto- and iso-agglutinins intact.

The following observation furnishes additional proof of the capacity of erythrocytes to absorb hæmagglutinin in the cold. Specimens of the blood of a number of infected animals were collected in sterile tubes and placed immediately in the ice chest to clot. After six hours the sera were separated from the clots by centrifugalisation and were examined for auto- and iso-agglutinins. In several cases the results were negative, no auto- or iso-agglutination being observed. In other instances the red cells were found to be clumped to a greater or less degree. The amount of agglutinin present in these sera was then compared with that occurring in the citrated plasma of the same animals which had been separated from the red cells at 37° C. The following procedure was adopted:—The serum and plasma were diluted with gradually increasing amounts of 0·9-per-cent. NaCl solution, and the degree of dilution observed at which they no longer caused complete agglutination of a given volume of the red cell suspension in a stated time.

The results obtained with one of the animals (Rabbit 1035, infected with *T. brucei*) are given in tabular form. It is to be observed that there was at least five or six times as much agglutinin in the plasma which had been separated from the red cells at 37° C. as in the serum obtained from blood which had been allowed to clot in the ice box.

The sera of the other animals all showed a considerable deficiency in agglutinin as compared with that present in the plasma of the same animals.

When examining the blood for auto-agglutination it was found that the strongest reactions were obtained by dropping the blood into a very small quantity of warm citrated saline solution, and then separating the plasma from the red cells as speedily as possible with the centrifuge. A perhaps even more satisfactory method, and one in which the dilution of the plasma by the citrate is avoided, is to use the defibrinated plasma obtained by shaking the blood at a sustained temperature of between 37° and 40° C. in a bottle containing a few glass beads.

Well marked agglutination was frequently observed when the plasma obtained in this manner was added to a 5-per-cent. suspension of red cells, whilst either a negative or only slightly positive result was obtained with the serum derived from blood which had clotted at laboratory temperature or in the ice chest.

Reversibility of the Reaction.—In view of the fact that red cells absorb agglutinin to a much greater extent at low temperatures and only slightly

Table III.—Diminution of Amount of Auto- and Iso-agglutinin in Serum obtained from Blood which was allowed to Clot at 0° C.

Equal volumes of red cell suspension and diluted serum or plasma used. Temp. of experiment 0° C.		
5 per cent. suspension of washed red cells of Rabbit 1035 (infected with *T. brucei*) and of normal rabbit.	Serum of Rabbit 1035 obtained by allowing the blood to clot at 0° C. Plasma of Rabbit 1035 separated from the red cells at 37° C.	Result.
Rabbit 1035	Undiluted serum	Complete auto-agglutination in 15 mins.
	„ plasma	„ „ 10 „
Normal rabbit	„ serum	Complete iso-agglutination 10 „
	„ plasma	„ „ 10 „
Rabbit 1035	Serum diluted with an equal volume of 0·9 per cent. NaCl solution	Complete auto-agglutination in 20 „
	Plasma „ „ „	„ „ 10 „
Normal rabbit	Serum „ „ „	Complete iso-agglutination 10 „
	Plasma „ „ „	„ „ 10 „
Rabbit 1035	Serum diluted with 2 volumes of 0·9 per cent. NaCl solution	Complete auto-agglutination in 30 „ *
	Plasma „ „ „	„ „ 15 „
Normal rabbit	Serum „ „ „	Complete iso-agglutination 15 „
	Plasma „ „ „	„ „ 10 „
Rabbit 1035	Serum diluted with 3 volumes of 0·9 per cent. NaCl solution	Partial auto-agglutination in 30 „
	Plasma „ „ „	Complete „ 30 „
Normal rabbit	Serum „ „ „	Complete iso-agglutination 20 „ †
	Plasma „ „ „	„ „ 20 „
Rabbit 1035	Serum diluted with 4 volumes of 0·9 per cent. NaCl solution	Trace auto-agglutination in 30 mins.
	Plasma „ „ „	Complete „ 30 „
Normal rabbit	Serum „ „ „	Very marked iso-agglutination 30 „
	Plasma „ „ „	Complete „ 20 „
Rabbit 1035	Serum diluted with 6 volumes of 0·9 per cent. NaCl solution	No agglutination in 30 mins.
	Plasma „ „ „	Complete auto-agglutination 30 „
Normal rabbit	Serum „ „ „	Slight iso-agglutination 30 „
	Plasma „ „ „	Complete „ 20 „
Rabbit 1035	Serum diluted with 9 volumes of 0·9 per cent. NaCl solution	No agglutination in 30 „
	Plasma „ „ „	Complete auto-agglutination 30 „
Normal rabbit	Serum „ „ „	No iso-agglutination 30 „
	Plasma „ „ „	Complete iso-agglutination 20 „
Rabbit 1035	Plasma diluted with 12 volumes of 0·9 per cent. NaCl solution	Complete auto-agglutination in 30 „ ‡
Normal rabbit	„ „ „ „	„ iso-agglutination 30 „
Rabbit 1035	Plasma diluted with 14 volumes of 0·9 per cent. NaCl solution	Partial auto-agglutination in 30 „
Normal rabbit	„ „ „ „	Complete iso-agglutination 30 „ §
Rabbit 1035	Plasma diluted with 19 volumes of 0·9 per cent. NaCl solution	No agglutination in 30 „
Normal rabbit	„ „ „ „	Slight iso-agglutination 30 „

* Denotes the end point of the auto-reaction of serum.
‡ „ „ „ plasma.
† Denotes the end point of iso-reaction of serum.
§ „ „ „ plasma.

at higher temperatures (37° to 40° C.), the question arises as to whether the absorption of agglutinin belongs to the group of reactions which have been designated "reversible."* In other words, will raising the temperature of the agglutinated masses of red cells cause the clumps to disintegrate into their corpuscular elements?

Experiment.—One volume of red corpuscles of Rabbit 1022 (infected with *T. gambiense*) was added to 20 volumes of the defibrinated plasma of the same animal. After an hour's sojourn in the ice chest complete agglutination of the erythrocytes was found to have occurred. After stirring the clumps up thoroughly with a glass rod a small drop of the suspension was placed on a cover-slip and a hanging drop preparation made. On examining with the microscope large masses of agglomerated red blood cells and also considerable rouleaux formation were seen. The mixture of clumped red cells and defibrinated plasma was now placed in the incubator at 37° C. In about 15 minutes the clumps were no longer visible, and the erythrocytes appeared to be evenly suspended throughout the fluid. A hanging drop preparation was made on a warm slide and cover-slip. As long as the temperature was maintained at 37° C., there was no tendency to agglutination. When the suspension was again cooled to 0° C., agglutination of the red cells reappeared after a few minutes.

From this and similar experiments it follows that the reaction is reversible, the phenomenon disappearing on warming and reappearing on cooling.

Auto-agglutination in the Blood of Normal Animals.—Attention has already been drawn to the fact that a certain amount of auto-agglutination was frequently observed in the control tests of normal blood. Some years ago Klein† found auto-agglutinin to be present in the serum of a number of normal horses.

Landsteiner‡ demonstrated the existence of a similar substance in the blood of rabbits, horses, dogs, and cattle.

Other writers, on the contrary, deny the existence of auto-agglutinin in normal blood. Dudgeon§ in a recent paper states that auto-agglutination does not occur in normal human blood.

It was decided to re-investigate this subject more fully, using the blood of considerable number of normal animals of different kinds.

* Arrhenius, 'Immuno-Chemistry,' ch. 2.

† 'Beiträge zur Kenntniss der Agglutination rother Blutkörperchen," 'Wien. Klin. Woch. 1902, No. 16, p. 413.

‡ "Ueber Beziehungen zwischen dem Blutserum und den Körperzellen," 'Münch. Med. Woch.,' 1903, No. 42.

§ "On the Presence of Hæmagglutinins, etc., in the Blood obtained from Infectious and Non-Infectious Diseases in Man," 'Roy. Soc. Proc.,' 1909, B, vol. 81, p. 207.

Technique.—The blood was obtained from a convenient vein, and the defibrinated plasma separated from the red corpuscles at 37° C. in the manner already described. The red corpuscles were washed three times in warm saline solution, and finally a 5-per-cent. suspension made in 0·9-per-cent. sodium chloride solution. Equal volumes of the defibrinated plasma and red cell suspension were drawn up together into three fine pipettes which were then subjected to a temperature of 0°, 15°, and 37° C. respectively. The pipettes were kept in the vertical position, and the contents examined for auto-agglutination with the aid of a lens from time to time. It was found in the majority of cases that the test could not well be continued for longer than one hour, owing to the fact that in most cases the erythrocytes had subsided to a marked degree after the lapse of this period. At times the citrated plasma was substituted for the defibrinated plasma. The same precautions regarding temperature were taken, and only very small amounts of citrate solution (not more than a tenth of the volume of plasma) employed. The plasma obtained in this way frequently clotted, but the process was sufficiently retarded to permit of the previous separation of the red corpuscles. No appreciable difference between the agglutinating action of the defibrinated and citrated plasma was observed.

The results of this investigation of normal blood for auto-agglutinin may be summarised by stating that small quantities of auto-agglutinin were found to be present in the blood of rabbits (14), guinea-pigs (4), goats (3), dogs (2), horses (4), donkeys (2), monkeys (*Macacus rhesus*) (2), and *Cercopithecus callitrichus* (2). Sometimes, especially in goats and guinea-pigs, the amount present was exceedingly small, and considerable care was necessary to demonstrate its existence. In these cases a larger volume of serum was used in proportion to the amount of red cells and the reaction allowed to proceed for a longer period. It is to be observed that clumping of the erythrocytes only occurred in the tests carried out at low temperatures, and not in those subjected to a temperature of 37° C.

Relation of Auto-agglutinin of Normal Blood to that present in the Blood of Animals infected with Trypanosomes.—In this connection it may be remarked that in the blood of infected animals there exists a considerable excess of auto-agglutinin beyond that present in the blood of normal animals. It was found that diluting the defibrinated plasma of normal blood with twice its volume of normal saline solution usually sufficed to destroy its agglutinating action. On the other hand, it was often possible to dilute the infected plasma 15- or 20-fold, and still obtain complete agglutination of the erythrocyte suspension.

Effect of Heat on Auto-agglutinin.—Different portions of the defibrinated

plasma of normal and infected animals were heated in a water bath to 58° C. and 70° to 72° C. respectively for 20 minutes. Heating to 58° C. was found not to destroy auto-agglutinin, whereas plasma which had been subjected to a temperature of 70° C. for 20 minutes had completely lost this property.

Significance of the Phenomenon in Trypanosomal Infections.—The question of the mechanism of production of auto-agglutination in trypanosomal infections is one which has frequently been discussed, but as yet no satisfactory explanation has been offered. With reference to this question it appears to me that two theories might be advanced to explain the development of an excess of auto-agglutinin in this disease.

It has long been recognised that the blood of men and the lower animals suffering from trypanosomiasis is frequently very anæmic. Both the percentage of hæmoglobin and the number of red corpuscles per cubic millimetre fall to a low level. This is particularly the case in the last stages of the disease. Conceivably auto-agglutinin might develop in the plasma as a result of auto-inoculation of an animal resulting from the destruction of its own erythrocytes.

There are, however, many considerations which operate against this view. In the first place I have found no constant relation between the development of anæmia and auto-agglutination of the red cells. By the aid of systematic hæmocrit examinations of the blood of recently infected animals it was observed that auto-agglutination was usually pronounced for a considerable period before any marked fall of the hæmocrit value had occurred. Secondly, a marked degree of auto-agglutination comparable to that occurring in trypanosomiasis has not been described in any other of the diseases in which anæmia is a distinctive feature. Dudgeon* examined the blood of 26 cases of anæmia due to various causes without finding a single example of auto-agglutination. It is doubtful, however, whether the technique adopted by Dudgeon is suitable for the recognition of small amounts of auto-agglutinin. Then again it is generally recognised as impossible to evoke the production of auto-bodies experimentally by inoculating an animal with its own tissues.

Experiment.—A rabbit was injected intraperitoneally with 10 c.c. of its own erythrocytes which had been laked with distilled water and the resulting solution made isotonic with sodium chloride. This injection was repeated after an interval of a week. No increase of auto-agglutinin was found to occur in the animal's blood.

Another possible explanation for the cause of the development of auto-

* "On the Presence of Hæmagglutinins, etc., in the Blood obtained from Infectious and Non-infectious Diseases in Man," 'Roy. Soc. Proc.,' 1909, B, vol. 80, p. 531.

agglutinin is that it is formed by the animal mechanism as a direct response to the stimulus of the pathogenic agent. In the consideration of this question it is necessary to inquire whether auto-agglutinin alone is present in excess in infected blood or whether we have at the same time a corresponding alteration in the iso- and hetero-agglutinin contents of the serum.

Mention has already been made of the fact that in every case where the serum of an infected animal was found to possess the property of clumping its own erythrocytes to a considerable degree, it also agglutinated markedly those of other members of the same species.

It was further observed that the plasma of infected animals frequently appeared to agglutinate the red cells of animals belonging to different species to a greater extent than normal. The case of human trypanosomiasis already referred to presented an excellent example of an increased capacity on the part of an infected serum to agglutinate foreign erythrocytes. The serum of this case clumped the red corpuscles of rats, guinea-pigs and rabbits in a remarkable manner. A few drops of the inactivated serum when added to an equal volume of the blood of one of these animals caused intense agglutination in a few seconds at room temperature. The action of normal human sera on these corpuscles was much slower and did not approach that of the former in intensity.

Experiments were undertaken with the object of comparing quantitatively the auto-, iso-, and hetero-agglutinin in the blood of several infected animals with that existing in the blood of normal animals of the same kind.

Technique.—The method adopted was that previously used for comparing the amount of auto- and iso-agglutinin in the defibrinated plasma separated from the red cells at 37° C. with that present in the serum obtained from blood which had clotted at 0° C.

Inactivated defibrinated plasma was prepared from normal Rabbit 1 and from Rabbit 896 (infected with *T. dimorphon*), and a 5 per cent. suspension of washed erythrocytes from the following animals: normal Rabbits 1 and 2, Rabbit 896 and a normal horse and guinea-pig.

From the results of this experiment, details of which are given in Table IV, and from other observations of the same kind one is led to conclude that in the blood of infected animals, in addition to an excess of auto-agglutinin, there is also frequently a corresponding increase in iso- and hetero-agglutinin.

The question now arises as to whether these reactions are manifestations of the same body or of different specific agglutinins. The procedure usually adopted for the solution of problems of this nature is the saturation of a portion of the serum by the red cells of one of the varieties in

Table IV.—Comparison of the Amount of Auto-, Iso-, and Hetero-agglutinin in the Plasma of Rabbit 896 (infected with *T. dimorphon*) and Normal Rabbit 1.

Equal volumes of red cell suspension and diluted plasma used. Temperature of experiment 0° C.		
5 per cent. suspension of washed erythrocytes in normal saline solution.	Plasma of Rabbits 1 and 896 diluted with increasing amounts of normal saline solution.	Result.
Rabbit 1	Rabbit 1 undiluted.....	Complete agglutination in 20 mins.
„ 1	„ 896 „	„ „ 20 „
„ 2	„ 1 „	„ „ 20 „
„ 2	„ 896 „	„ „ 20 „
„ 896	„ 1 „	„ „ 20 „
„ 896	„ 896 „	„ „ 20 „
Horse	„ 1 „	„ „ 10 „
	„ 896 „	„ „ 10 „
Guinea-pig	„ 1 „	Marked „ 45 „
	„ 896 „	Complete „ 45 „
Rabbit 1	„ 1 diluted with twice its vol. of 0·9 p. c. NaCl solution	Nil „ 45 „
„ 1	„ 896 „	Complete „ 20 „
„ 2	„ 1 „	Slight „ 20 „
„ 2	„ 896 „	Complete „ 20 „
„ 896	„ 1 „	Nil „ 20 „
„ 896	„ 896 „	Complete „ 20 „
Horse	„ 1 „	Marked „ 45 „
	„ 896 „	Complete „ 15 „
Guinea-pig	„ 1 „	Nil „ 45 „
	„ 896 „	Complete „ 15 „
Rabbit 1	„ 1 diluted with 4 times its vol. of 0·9 p. c. NaCl solution	Nil „ 15 „
„ 1	„ 896 „	Complete „ 15 „
„ 2	„ 1 „	Nil „ 15 „
„ 2	„ 896 „	Complete „ 30 „
„ 896	„ 1 „	Nil „ 30 „
„ 896	„ 896 „	Complete „ 30 „
Horse	„ 1 „	Slight „ 30 „
	„ 896 „	Complete „ 30 „
Guinea-pig	„ 1 „	Nil „ 30 „
	„ 896 „	Complete „ 20 „
Rabbit 1	„ 896 diluted with 6 times its vol. of 0·9 p. c. NaCl solution	„ „ 20 „
„ 2	„ 896 „	„ „ 30 „
„ 896	„ 896 „	„ „ 30 „
Horse	„ 896 „	Marked „ 45 „
Guinea-pig	„ 896 „	Complete „ 15 „
Rabbit 1	„ 896 diluted with 9 times its vol. of 0·9 p. c. NaCl solution	„ „ 20 „
„ 2	„ 896 „	Partial „ 45 „
„ 896	„ 896 „	Complete „ 30 „
Horse	„ 896 „	Trace „ 45 „
Guinea-pig	„ 896 „	Complete „ 15 „
Rabbit 1	„ 896 diluted with 14 times its vol. of 0·9 p. c. NaCl solution	Slight „ 45 „
„ 2	„ 896 „	Nil „ 45 „
„ 896	„ 896 „	Slight „ 45 „
Horse	„ 896 „	Nil „ 45 „
Guinea-pig	„ 896 „	Slight „ 45 „

question, and then, after allowing reaction to take place for some hours, centrifugalising and examining the extracted serum regarding its agglutinating action on the red cells of the kind used for extraction and also on the other varieties of erythrocytes.

Malkoff,* adopting this technique, arrived at the conclusion that there exist in goat's serum, which is capable of agglutinating the erythrocytes of many kinds of animals, different specific agglutinins, each of which has a specific affinity for the corresponding variety of red cells.

Experiment.—(1) One volume of defibrinated plasma of Rabbit 896 (infected with *T. dimorphon*) was extracted for 12 hours at 0° C. with an equal volume of the undiluted red blood cells of the same animal. (Extracted plasma A.) (2) Here the proportion of plasma to erythrocytes was one to five. (Extracted plasma B.) (3) One volume of the same plasma was treated with one volume of normal horse's red cells. (Extracted plasma C.)

A 5-per-cent. suspension of washed erythrocytes was prepared from the following animals: Rabbit 896, normal Rabbit 1, normal Donkeys 1 and 2, a normal guinea-pig, horse, *Macacus rhesus*, *Cercopithecus callitrichus*, and human being.

The results indicate that complete extraction of the infected plasma of Rabbit 896 by its own erythrocytes and those of a normal horse does not completely destroy the agglutinating action of the plasma on the red blood cells of other animals, although it is to be noted that in most cases when the plasma had been extracted with five times its volume of its own red cells there was a marked lessening or even total disappearance of this action. This diminution of the agglutinating action of the plasma cannot be explained by mere dilution with the small amount of saline solution adhering to the red cells, as the plasma still caused marked agglutination after the addition of fifteen times its volume of 0·9 per cent. NaCl solution.

It is doubtful, however, whether experiments of this kind really have the importance that has been assigned to them by Malkoff and others.

Landsteiner and Sturli† using normal horse and dog serum and 11 varieties of erythrocytes, confirmed Malkoff's observation that saturation of the serum with one kind of red blood cell deprived it of the power to agglutinate this variety, and this only. They furthermore showed that red cells which had already been once completely agglutinated were still able to react with another kind of serum, and that the new serum after the reaction had lost its power to agglutinate fresh corpuscles of the same kind. Hence, as

* "Beiträge zur Frage der Agglutination von rother Blutkörperchen," 'Deutsche Med. Woch.,' 1900, No. 14.

† "Ueber die Hämagglutinine normaler Sera," 'Wien. Klin. Woch.,' 1902, p. 38.

Table V.—Agglutinating Action of Plasma which had been previously Extracted with Red Blood Cells.

Equal volumes of red cell suspension and plasma used. Temp. of experiment 0° C.		
5 per cent. suspension of washed erythrocytes in normal saline solution.	Untreated plasma of Rabbit 896 (infected with *T. dimorphon*) and also plasma of the same animal extracted with red cells as follows: A, with an equal volume of red blood cells of Rabbit 896. B, with five times its volume of red cells of Rabbit 896. C, with an equal volume of red cells of a normal horse.	Result.
Rabbit 896	Untreated plasma	Complete agglutination in 10 mins.
	Extracted „ A	Partial „ 45 „
	„ „ B	No „ 45 „
	„ „ C	Complete „ 15 „
Rabbit 1	Untreated plasma	Complete „ 15 „
	Extracted „ A	„ „ 30 „
	„ „ B	No „ 45 „
	„ „ C	Complete „ 15 „
Donkey 1	Untreated plasma	Complete „ 15 „
	Extracted „ A	Almost complete „ 45 „
	„ „ B	No „ 45 „
	„ „ C	Marked „ 45 „
Donkey 2	Untreated plasma	Complete „ 15 „
	Extracted „ A	„ „ 30 „
	„ „ B	Trace „ 45 „
	„ „ C	Complete „ 45 „
Guinea-pig	Untreated plasma	Complete „ 15 „
	Extracted „ A	„ „ 15 „
	„ „ B	Partial „ 45 „
	„ „ C	Complete „ 15 „
Horse	Untreated plasma	Complete „ 10 „
	Extracted „ A	„ „ 15 „
	„ „ B	Slight „ 45 „
	„ „ C	Partial „ 45 „
Macacus rhesus	Untreated plasma	Complete „ 15 „
	Extracted „ A	„ „ 30 „
	„ „ B	Slight „ 45 „
	„ „ C	Complete „ 30 „
Cercopithecus callitrichus	Untreated plasma	Complete „ 15 „
	Extracted „ A	„ „ 15 „
	„ „ B	„ „ 30 „
	„ „ C	„ „ 15 „
Human being	Untreated plasma	Complete „ 10 „
	Extracted „ A	„ „ 20 „
	„ „ B	Marked „ 45 „
	„ „ C	Complete „ 15 „

Landsteiner points out, the problem had assumed a very complex aspect, the enormous number of specific agglutinins in normal serum appearing uneconomic.

Landsteiner and Sturli suggest another hypothesis to explain these facts, namely, that during the process of agglutination some substance passes from the red cell to the serum, and that after complete agglutination the serum, in consequence of the combination, agglutinin + corpuscle substance, can no longer react with red cells of the same kind, but can with those of other animals. By this theory they maintain that the facts can be explained without the necessity for assuming the presence of an enormous number of differently acting substances or groups of substances in normal serum.

A certain amount of support is afforded this view by the observation of Landsteiner that a watery extract of the corpuscles of a turkey, when added to horse serum, almost completely prevents its agglutinating action on the red cells of the turkey, but only in a very slight degree on other kinds of blood. This last observation was subsequently confirmed by Lazar.* However, unless all traces of the stromata of the red cells had been removed from the hæmoglobin solution—and this is by no means an easy performance—an obvious explanation for this inhibiting action of such solutions would be that the stromata themselves had fixed the agglutinins present in the horse serum, and consequently there would be little, if any, left to act upon the red corpuscles. Naturally, in this case, the inhibiting action would be specific for the variety of red cells from which the hæmoglobin solution was made.

The fact that the phenomenon of auto-agglutination is reversible allows one to approach the subject of specificity from a different point of view, namely, by extracting the completely agglutinated red cells with a small quantity of normal saline solution at 37° C., and then investigating the nature of the digest.

Experiment.—To 10 c.c. of defibrinated plasma of Rabbit 1035 (infected with *T. brucei*) were added 0·2 c.c. of the red cells of the same animal. After allowing the mixture to stand in the ice chest for 12 hours with occasional stirrings the supernatant plasma was decanted off, and the clumped red blood cells washed four times with at least 10 times their volume of normal saline solution at 0° C.; 0·2 c.c. of normal salt solution was then added to the agglutinated mass of red cells and the mixture allowed to digest at 40° C. for half an hour. At the end of this time no trace of agglutination was

* "Ueber die Bedeutung der lipoiden Stoffe der rothen Blutkörperchen für den Mechanismus der Agglutination," 'Wien. Klin. Woch.,' 1905, p. 1012.

visible. The red cells were then quickly thrown down by centrifugalisation and the supernatant fluid removed (Digest solution).

A 5-per-cent. suspension of red blood cells in normal saline solution was prepared from the following animals: Rabbit 1035 (infected with *T. brucei*), normal Rabbit A, Donkey 2 (infected with *T. rhodesiense*), normal Donkey A, Goat 1041 (infected with *T. rhodesiense*), normal Goat A, and from a normal rat, guinea-pig, dog, horse, *Macacus rhesus*, *Cercopithecus callitrichus*, and human being.

Table VI.—Agglutinating Action of a Solution Obtained by Digesting Auto-agglutinated Red Blood Cells with Normal Saline Solution at 40° C.

Equal volumes of red cell suspension and defibrinated plasma or digest solution used. Temperature of experiment 0° C.		
5 per cent. suspension of washed erythrocytes in normal saline solution.	Untreated defibrinated plasma of Rabbit 1035 (infected with nagana), and solution obtained by digesting the auto-agglutinated red cells of the same animal with normal saline solution at 37° C.	Result.
Rabbit 1035 (infected with *T. brucei*)	Defibrinated plasma	Complete agglutination in 10 mins.
	Digest solution	,, ,, 10 ,,
Normal Rabbit A	Defibrinated plasma	,, ,, 10 ,,
	Digest solution	,, ,, 10 ,,
Donkey 2 (infected with *T. rhodesiense*)	Defibrinated plasma	,, ,, 10 ,,
	Digest solution	,, ,, 30 ,,
Normal Donkey A	Defibrinated plasma	,, ,, 15 ,,
	Digest solution	Slight ,, 45 ,,
Goat 1041 (infected with *T. rhodesiense*)	Defibrinated plasma	Complete ,, 10 ,,
	Digest solution	No ,, 45 ,,
Normal Goat A	Defibrinated plasma	Complete ,, 10 ,,
	Digest solution	No ,, 45 ,,
Rat	Defibrinated plasma	Complete ,, 15 ,,
	Digest solution	,, ,, 20 ,,
Guinea-pig	Defibrinated plasma	,, ,, 15 ,,
	Digest solution	,, ,, 20 ,,
Dog	Defibrinated plasma	,, ,, 5 ,,
	Digest solution	,, ,, 10 ,,
Horse	Defibrinated plasma	,, ,, 10 ,,
	Digest solution	Partial ,, 45 ,,
Macacus rhesus	Defibrinated plasma	Complete ,, 15 ,,
	Digest solution	,, ,, 30 ,,
Cercopithecus callitrichus	Defibrinated plasma	,, ,, 15 ,,
	Digest solution	,, ,, 15 ,,
Human being	Defibrinated plasma	,, ,, 10 ,,
	Digest solution	,, ,, 15 ,,

N.B.—The four specimens of normal saline solution which had been used for washing the clumped red cells were also examined. With the exception of occasional traces in the first, no agglutinin was found in these solutions.

The capacity of the untreated plasma of Rabbit 1035 and of the solution prepared by digesting the agglutinated red cells with normal saline at 0° C. to agglutinate these different erythrocytes was then examined.

The information obtained from observations of this kind is extremely interesting. In the experiment recorded a substance was extracted from the auto-agglutinated erythrocytes of a rabbit infected with *T. brucei* which clumped not only its own erythrocytes and those of other rabbits, but also the red cells of many other animals of different species. In other words it would appear that the auto-agglutinin is not a body equipped with a high degree of specificity, but that it can also act as iso- and hetero-agglutinin on the erythrocytes of other rabbits and those of animals of widely different species.

Value of the Phenomenon as a Diagnostic Sign.—Before discussing this question it is necessary to emphasise the importance of careful observation in determining whether a certain blood really agglutinates or not. So far as can be gathered from the papers in which the existence of the phenomenon in trypanosomiasis has been recorded, it has been invariably decided from the examination of cover-slip preparations of the blood. Although a considerable degree of auto-agglutination is easily recognised in a well-made cover-slip preparation, yet it is often extremely difficult, or even impossible, to decide whether the red cells are really agglutinated when the phenomenon is not so distinct. A certain amount of massing together of the erythrocytes is frequently evident at the edges of even the best cover-slip preparations of normal blood, whereas if the slide and cover-slip be not perfectly clean the red cells are found to be anything but evenly distributed, but are grouped together into little masses and rouleaux, separated from one another by plasma—an appearance closely resembling that to be observed in infected blood when the amount of auto-agglutination is slight. On the other hand, a slight degree of auto-agglutination can be easily obscured by pressure on the cover-slip resulting in the separation of the erythrocytes one from the other.

Furthermore, it has been shown that small amounts of auto-agglutinin exist constantly in the blood of many normal animals. In horses and donkeys auto-agglutinin is sometimes present in such an extent as to give rise to a more or less characteristic appearance in cover-slip preparations. This is specially the case when the preparations are made out of doors at a somewhat low temperature. However, I have never observed in cover-slip preparations of the blood of normal animals a condition approaching in intensity the well-marked clumping obtaining in infected cases. When a high degree of auto-agglutination exists the corpuscles are seen to have

become agglomerated into tight clumps, the outlines of the individual cells being indistinct, or even completely lost, so that the clumps appear to consist of red cells which have fused together into a homogeneous mass. In order to evoke as characteristic an appearance as possible the preparation should be made at the lowest temperature practicable.

The next point to be considered is whether auto-agglutination is a constant feature in trypanosomal infections.

Martin, Lebœuf, and Roubaud* stated that in the large number of cases of human trypanosomiasis examined by them in the French Congo, auto-agglutination was always present. In the tables appearing in their report, the condition of the blood as regards auto-agglutination is indicated by numbers from 0 to 10, the cipher meaning that there is no agglutination, whereas the greatest degree of agglutination is indicated as 10; the intermediate figures denote intermediate degrees of agglutination.

In view of the technique used by them—the mere examinations of cover-slip preparations of the fresh blood—such a classification appears to be a somewhat unwarrantable refinement.

Todd† in a recent paper classifies as regards auto-agglutination a large number (1406) of cases examined by Dutton and himself in the Congo Free State. Of the 395 cases in which auto-agglutination was present, trypanosomes were found in only 183. However, as Todd himself states, probably because of the insufficient search for them (the cases were seen and examined on one occasion only), trypanosomes were present much more often than they were found.

Later in the same paper it is stated that only in three cases were trypanosomes not present when an extremely well marked auto-agglutination was recorded. One of these was a case of relapsing fever; another was a much emaciated marasmic individual, and the third was a case of syphilis.

Regarding the frequency of the phenomenon in the blood of experimentally infected animals, it need only be stated that as a rule auto-agglutination is best marked in the blood of the larger animals, *e.g.* horse and donkey. It is usually also very distinct in the monkey, dog, rabbit, and goat. In the rat, mouse, and guinea-pig it is generally slight or absent.

In the ordinary course of events the infected animal shows parasites in its blood for some time before a distinct auto-agglutination develops. Occasionally, however, auto-agglutination appears before trypanosomes have been

* 'Rapport de la Mission d'Études de la Maladie du Sommeil au Congo Française,' 1906—8, p. 281.

† "A Note on the Occurrence of Auto-agglutination of the Red Cells in Human Trypanosomiasis," 'Bull. Soc. Path. Exot.,' 1910, p. 438.

found in the blood. I have had under observation a goat, infected with *T. gambiense*, in which trypanosomes have never been seen in the blood, but in which a well marked auto-agglutination had developed. The blood was shown to be infective by injection into rats.

The last point to be decided in considering the value of the phenomenon as a diagnostic sign is whether it occurs in other diseases besides trypanosomiasis. In addition to the three cases mentioned by Todd, auto-agglutination has occasionally been noted in persons suffering from other diseases than Sleeping Sickness.

Klein,* in 1890, found auto-agglutination of the red blood cells in a case of hepatic cirrhosis (Hanot). Dudgeon† mentions the case of a West Indian negro which was considered to be one of tertiary hepatic syphilis, where the blood exhibited spontaneous clumping. He also found auto-agglutinin in the blood of a case of long standing epilepsy. Martin and Darré‡ assert that the phenomenon is to be met with in certain forms of icterus due to hæmolysis.

Quite recently Nattan-Larrier§ described the existence of auto-agglutination in rats infected with the *Spirilla obermierei*. It is interesting to note in this connection that of Todd's three cases which exhibited well marked auto-agglutination, but were not infected with trypanosomes, one was a case of relapsing fever and another a case of syphilis, as was also the only definite instance of the phenomenon seen by Dudgeon. It is of importance to know whether auto-agglutination is often present in spirochaetal infections.

In conclusion it may be stated that in the light of the information obtainable a well-marked degree of auto-agglutination of the red blood cells is an extremely rare occurrence, apart from infection with trypanosomes.

Summary.

Auto- and iso-agglutinin are present in the blood of cases of Sleeping Sickness and of animals infected with trypanosomiasis.

Reaction between auto-agglutinin and red blood cells takes place only at low temperatures.

Auto-agglutinin can be removed from plasma by absorption with the erythrocytes of the same animal at 0° C.

The reaction between auto-agglutinin and red blood cells is reversible.

* "Ueber die Untersuchung der Formelemente des Blutes und ihre Bedeutung für die praktische Medicin," 'Wien. Klin. Woch.,' 1890, Nos. 36—40.

† *Loc. cit.*

‡ 'Bull. et Mémoires Soc. Méd. des Hôpitaux de Paris,' 1909, p. 599.

§ "L'Autoagglutination des Hématies dans la Spirillose Expérimentale," 'Bull. Soc. Path. Exot.,' 1910, p. 425.

Auto-agglutinin exists in small amounts in the blood of many normal animals.

Auto-, iso- and hetero-agglutinin are frequently present in much greater amount in the blood of infected animals than in that of normal animals, and it is due to this fact that clumping of the red blood cells is often visible in fresh cover-slip preparations of the blood of infected animals.

From the red blood cells of an infected animal which have been agglutinated in the cold by the plasma of the same animal an active substance can be extracted with normal saline solution at 37° C.

This substance agglutinates not only the red cells of the same animal and other members of the same species, but also those of many animals of different species.

It is to be inferred from the information at present available that a marked degree of auto-agglutination of the red blood cells is an extremely rare occurrence apart from an infection with trypanosomes.