CONTRIBUTIONS TO THE STUDY OF HEMAGGLUTININS AND HEMOLYSINS.[1]

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While it is now fully established that the injection of the blood corpuscles of one species of animal into another species causes the production, by the latter, of agglutinins and lysins specific for the erythrocytes of the former, yet the part played by the different portions of the red corpuscle, stroma, hemoglobin, or cellular contents, in the causation of these phenomena, has not yet been definitely settled. This problem has been the subject of investigation by van Dungern, and by several members of the French School of Bacteriologists, notably Bordet, Nolf, and Leblanc, without, however, yielding absolutely conclusive results.

Van Dungern,[2] experimenting with hen's blood, prepared laked blood and stroma by subjecting the corpuscles to the action of water mixed with a little ether. The resulting mixture was then filtered, the stroma collected and washed with water and ether. The dried residue was now dissolved in dilute HCl or in five per cent $MgSO_4$. Both laked blood and stroma solution were injected into animals without causing any changes in their blood serum. From van Dungern's experiments he concluded that the substances producing specific antibodies existed in the blood cell in such a labile condition that they were destroyed by such simple procedures as the addition of water to the corpuscle.

In France Bordet[3] assigns to the stroma the chief function in the production of hemolysis, attributing no definite

[1] The cost of these investigations was borne by the Rockefeller Institute for Medical Research. Received for publication Jan. 17, 1904.

[2] Van Dungern. Munchener Medicinische Wochenschrift, Vol. 46, 1889, 1, p. 449.

[3] Bordet. Annales de l'Institut Pasteur, Vol. 14, 1900, p. 257.

properties to the laked portion of the blood, while Nolf,[1] on the contrary, found that animals treated with stroma yielded serum containing merely agglutinins, while the serum from animals treated with laked blood was endowed with hemolytic properties. This direct contradiction in the results found by Bordet and Nolf is partially explained when we compare the experiments of the two observers. Bordet used four to five cubic centimeters of rabbit's blood, which he laked with sterile water, and from which he obtained the stroma by centrifugation. With the laked portion of the blood he immunized one guinea-pig, and with the stroma another. After an interval of three weeks he killed these guinea-pigs, and found that the one treated with stroma furnished a strongly hemolytic serum, while the serum from the animal treated with laked blood possessed no specific properties.[2] Nolf selected for his experiments hen's blood, with the ingredients of which he immunized rabbits. After a number of injections he found that the serum from the rabbits treated with stroma agglutinated hen's erythrocytes in a dilution of 1-100 (.05 cubic centimeter serum for 5 cubic centimeters blood), while that from the rabbits treated with laked blood was strongly hemolytic, so much so that .5 centimeter serum completely dissolved one centimeter of blood.

Nolf's results, however, were not always free from contradiction. In several cases he noted that the serum from the rabbits treated with stroma, besides agglutinating hen's corpuscles, had a tendency to dissolve them in proportions greater than normal. In other cases he found that the serum from animals treated with laked blood, which was strongly hemolytic, also produced what he calls a trace of agglutination. Having observed, however, in the preparation of his materials, that small particles of stroma were present in the laked blood, and, conversely, traces of red coloring matter, that is, hemoglobin, were left clinging to the stroma, he concluded that in the animals treated with laked blood the

[1] Nolf. Annales de l'Institut Pasteur, Vol. 14, 1900, p. 297.

[2] It is impossible to see from Bordet's own account of his work that more than one injection was given, or more than one guinea-pig used.

traces of agglutination were due to these fine particles of stroma, the small amount of lysis present was due to the hemoglobin. Nolf concluded, therefore, that in the event of obtaining an absolutely pure stroma and an absolutely pure laked blood, the result of immunizing animals with these substances would be pure agglutination for the stroma and pure lysis for the laked blood.

Both Bordet and Nolf consider that the separation of the two phenomena, agglutination and lysis (in other words, the production of definite substances in the one case simply agglutinating the corpuscles without dissolving them, in the other case dissolving the corpuscles without agglutinating them), is made possible by laking the blood corpuscles with water and injecting aqueous solution and stroma respectively.

In Bordet's work it may be noted that he fails to state the strength of the lysis he artificially produced, and in the event of his having used but one injection, it must be remembered that the amount of lytic substances circulating in the normal blood serum is susceptible of great variation, and may be so great as to explain any observations taken after such preliminary treatment.

Finally, Leblanc[1] attempted to prepare pure hemoglobin, with which he immunized several animals. For this purpose he used ox blood which, after thorough washing, was laked with water containing a little ether. The stroma and globulins were precipitated from this mixture by half saturation with ammonium sulphate, after which the hemoglobin was thrown down by complete saturation with the same reagent. After injections for a month and a half with the hemoglobin so prepared, Leblanc found that the resulting serum gave an abundant precipitate with his hemoglobin solutions. He fails to state what effect, if any, this serum had upon the erythrocytes of the species selected as the source of the hemoglobin. Leblanc's hemoglobin was not obtained crystalline, and the presence of considerable amounts of albumin, both from the blood serum and from the albuminous content

[1] Leblanc. La Cellule, Vol. 18, 1901, p. 336.

of the red cell, cannot be overlooked. The precipitations he obtained may thus have been due to precipitins specific for the albumin injected, and not for the hemoglobin. The contradictions in the results of Bordet and Nolf and the imperfectly concluded experiments of van Dungern and Leblanc have furnished the basis for the investigations reported in this paper.

To make our results strictly comparable with those of Nolf and Bordet we employed rabbit's erythrocytes for the immunization of guinea-pigs, the combination used by Bordet, and hen's erythrocytes for the immunization of rabbits, that selected by Nolf. In the preparation of laked blood and stroma we followed in all cases the procedures recommended by the French observers. The blood was defibrinated in the usual manner and washed repeatedly with isotonic salt solution until the washings were quite free from serum. The corpuscles were then laked by the addition of three times their volume of distilled water. To avoid the formation of rouleaux, concentrated salt solution was now added until the content in salt of the entire mixture was brought up to one per cent. Upon centrifugalization the stroma was deposited as a fibrinous mass deeply tinged with hemoglobin. Before it can be obtained in a pure condition it must be repeatedly washed with normal salt solution, or with sterile water, which is afterwards made up to a one per cent solution by the addition of concentrated salt solution in appropriate amounts. We finally obtained in all cases stroma free from hemoglobin, which, when pure, consists of a thick, fibrinous, jelly-like mass, with an individual color for each species of animal, dull white for rabbits and pale yellow for hens.

The laked blood, containing hemoglobin and the albuminous and other ingredients of the red corpuscle which can be dissolved in water, was freed from stroma by long continued centrifugalization, lasting sometimes for two to three hours. The heavier part of the stroma was usually precipitated by this method after an hour's revolution. By changing the solutions to fresh tubes and again centrifugalizing, a very fine deposit of stroma was obtained. By repeatedly

transferring the solutions to new tubes, that point in the procedure when no more fine stroma is deposited may easily be observed, and at this point the laked blood was considered pure and was used for injections.

Both laked blood and stroma were prepared as aseptically as possible, and, considering the amount of manipulation required, our animals were singularly free from infections.

Finally we made the attempt to isolate pure crystalline hemoglobin and to immunize various animals with this substance, later testing the serum for agglutination and lysis for the blood corpuscles of the species of animals selected as the source of the hemoglobin, and for precipitation with these hemoglobin solutions.

Our hemoglobin was prepared from hen's and dog's blood by Hufner's method. The blood was first received in isotonic ammonium oxalate solution, centrifugalized, and washed repeatedly with normal salt solution until it was quite free from serum. It was then laked by the addition of a little ether and two to three times its volume of distilled water. From this mixture the hemoglobin was precipitated by the addition of alcohol up to twenty-five per cent. At 0° crystals of ox hemoglobin were deposited after twenty-four hours.

From dog's blood pure crystals may be obtained by this method with comparative ease, three or four recrystallizations being sufficient to obtain the hemoglobin in great purity. Owing to the ready solubility of hen's hemoglobin at temperatures even below 0°, and the consequent production of a large amount of mother liquor, the preparation of this body is rendered more difficult. Usually four or five recrystallizations are necessary to insure its purity. The hemoglobin when obtained in a crystalline condition was dissolved in water and used for injecting rabbits and guinea-pigs.

ANIMAL EXPERIMENTATION.

Our results become more accessible if we consider each set of animals separately, noting the effect of the injection of the various ingredients of the corpuscles upon each species.

A number of preliminary observations were made with serum drawn after three or four injections, but the amount of artificial lysis and agglutinins was not sufficiently above the range of normal to warrant any definite conclusions as to the source of these bodies. Moreover, these observations were by no means free from error, since we found that a freshly drawn serum might be so strongly hemolytic that the erythrocytes were almost immediately dissolved in the lower dilutions, before agglutination took place, and it thus became impossible to determine the exact limits of this phenomenon by following the series of changes from a partial to a complete precipitation. In many cases we were led to think, therefore, that we had been able to produce hemolysis without agglutination, looking upon the changes found in the higher dilutions as of the nature of " pseudo-agglutinations." The preparation of parallel series of dilutions, one set of which we placed on ice at a temperature of about 3° C., the other being placed in the thermostat at a temperature of 37° C., enabled us to study the limits of agglutination and lysis with accuracy. Furthermore, we made our observations only with animals so highly immunized that observation of the changes in their blood serum could not be regarded as accidental.

In the series of guinea-pigs treated with rabbit's laked blood and stroma, we found that the animals treated with *stroma* possessed a strongly hemolytic serum, a partial lysis being present even in a dilution of one to fifty, in accordance with the ideas of Bordet. This lysis, however, was always accompanied by agglutination and to the same degree of dilution, the agglutination being masked by the rapid solution of the corpuscles in the series of test-tubes placed in the thermostat. In those placed on ice, as well as in the mixture of erythrocytes and serum inactivated at 57° C., although no lysis took place, the agglutination was always present.

In the serum from animals treated with laked blood, moreover, definite specific substances could be demonstrated. Thus the serum produced after six injections of laked blood

caused complete agglutination in a dilution of one to twenty-five and a partial agglutination in a dilution of one to fifty. Only a trace of lysis was present in this serum, found in a mixture of .5 cubic centimeter serum to one cubic centimeter blood. This apparent discrepancy was explained by later experimentation,[1] the absence of lysis being due to lack of complement in this sera.

The following tables give the results of this series of observations:

TABLE I.

Series A, I.

Guinea-pig treated with rabbit's blood, laked.

Six injections, November 13 to November 28. Serum drawn December 1. Tested December 2.

5% Suspension Washed Blood.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.6 cc.	+	+
"	+	.5 cc.	+	Trace.
"	+	.1 cc.	+	0
"	+	.04 cc.	+	0
"	+	.02 cc.	Trace	0

[1] See Table 5, Series E, i., ii., iii., pp. 13, 14.

Series A, II.

Guinea-pig treated with rabbit's stroma.

Eight injections, October 15 to November 25. Serum drawn December 1. Tested December 2.

5% Suspension Washed Blood.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	+
"	+	.03 cc.	+	+
"	+	.025 cc.	+	Almost complete.
"	+	.02 cc.	+	Partial.
"	+	.015 cc.	Trace	Trace.
"	+	.01 cc.	0	0

Control[1] normal guinea-pig serum.

5% Suspension Washed Blood.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	0	0

[1] In all cases a number of normal animals were tested as controls.

In the series of animals immunized with hen's blood, laked, and stroma, we obtained results which were in part like those of Nolf. In our animals treated with laked blood, we found a strong lysis, even in dilutions of one to fifty, the lysis being always accompanied or preceded by agglutination in similar dilutions. At the same time the animals treated with stroma furnished a serum which was practically identical with that from the animals treated with laked blood, both agglutination and lysis being present in dilutions of one to fifty, partial agglutination and a trace of lysis being observable in dilutions of one to sixty-five. This would confirm the observations of Nolf as to the effect of the injection of stroma in the causation of agglutination, but would extend

the same observations by the demonstration of the presence of lysis also. At the same time a series of animals treated with the entire washed corpuscles, for the sake of comparison, gave, after five injections, serum almost identical with that obtained from the injection of the partial ingredients of the erythrocytes, complete agglutination in dilutions of one to fifty, with almost complete lysis in the same dilution, while a trace of lysis and a partial agglutination appeared in a dilution of one to sixty-five.

A further series of guinea-pigs treated with the laked blood and the stroma from hen's blood gave confirmatory results. The following tables explain these statements:

TABLE II.

Series B, I.

Rabbit treated with hen's blood laked.

Five injections, December 1 to December 13. Serum tested December 15.

5% Washed Hen's Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	+
"	+	.05 cc.	+	+
"	+	.02 cc.	+	+
"	+	.015 cc.	Partial	Partial.

Control normal rabbit serum.

5% Washed Hen's Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.5 cc.	+	+
"	+	.1 cc.	Trace	Trace.

Series B, II.

Rabbit treated with hen's stroma.

Six injections, December 1 to December 13. Serum drawn December 18. Tested December 19.

5% Washed Hen's Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	+
"	+	.025 cc.	+	+
"	+	.02 cc.	Almost complete	+
"	+	.015 cc.	Partial	Partial.
"	+	.01 cc.	Trace	Trace.

Control normal rabbit serum.

5% Washed Corpuscles.		Normal Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.5 cc.	+	+
"	+	.1 cc.	Trace	Trace.

Series B, III.

Rabbit treated with hen's blood entire.

Five injections, December 1 to December 13. Serum tested December 15.

5% Washed Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	+
"	+	.03 cc.	+	+
"	+	.02 cc.	Incomplete	+
"	+	.015 cc.	Trace	Partial.
"	+	.01 cc.	"	Trace.

Our observations on hen's blood were thus conclusive in showing that avian blood, at least one variety of it, when split up into its various ingredients and injected into rabbits or guinea-pigs, was capable of producing both agglutination and lysis in much the same way that the whole corpuscle acted. To determine whether all avian blood, or at least another variety of avian blood, had the same effect, a further series of observations was carried out with goose's blood. The stroma from this blood is easily obtained in considerable quantity, and after three washings is practically free from hemoglobin. It is rather deep yellow in color, consisting of finely divided particles easily injected subcutaneously or intraperitoneally. Large doses of both laked blood and stroma were used. A further series of animals were treated with the whole blood corpuscles washed free from serum, and another series with the washings from the stroma, which contained relatively little hemoglobin, but rather those portions of the stroma soluble in water.

The result of these experiments was most surprising. Every sample of serum examined showed a high degree of agglutination and practically no lysis, whether the serum was from animals treated with whole blood, laked blood, stroma, or second washings. No lysis was observable in any of these sera. At the same time the normal agglutination for goose's corpuscles by rabbit's serum was relatively high. A glance at the following table will make these points clear:

TABLE III.

Series C, I.

Rabbit immunized with washed goose corpuscles.

Seven injections, December 1 to December 17. Serum tested December 20.

5% Washed Goose Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	+
"	+	.05 cc.	+	+
"	+	.01 cc.	+	Faint.
"	+	.0075 cc.	+	Faint.
"	+	.005 cc.	+	Negative.
"	+	.002 cc.	+	
"	+	.001 cc.	+	
"	+	.00067 cc.	Trace.	
"	+	.0005 cc.	0	

Series C, II.

Control normal rabbit serum.

5% Washed Blood.		Normal Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.5 cc.	+	+
"	+	.2 cc.	+	0
"	+	.02 cc.	+	0
"	+	.01 cc.	+	0
"	+	.005 cc.	+	
"	+	.004 cc.	+	
"	+	.003 cc.	0	
"	+	.002 cc.	0	

NOTE. — Test for agglutination at 3° Centigrade, for lysis at 37° Centigrade. Both readings in same table for convenience.

Series C, III.

Rabbit treated with goose's blood laked.

Seven injections, December 1 to December 15. Serum tested December 20.

5% Washed Blood.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	Negative.
"	+	.01 cc.	+	"
"	+	.005 cc.	+	"
"	+	.002 cc.	+	
"	+	.001 cc.	Partial.	
"	+	.00067 cc.	0	

Series C, IV.

Rabbit tested with goose blood stroma.

Seven injections, December 1 to December 17. Serum tested December 20.

5% Washed Goose Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	Trace.
"	+	.05 cc.	+	Negative.
"	+	.01 cc.	+	"
"	+	.005 cc.	+	
"	+	.002 cc.	+	
"	+	.0015 cc.	Partial.	
"	+	.001 cc.	Trace.	

Series C, V.

Rabbit treated with second washing from goose blood.

Seven injections, December 1 to December 17. Serum tested, December 20.

5% Washed Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	o
"	+	.05 cc.	+	o
"	+	.01 cc.	+	o
"	+	.005 cc.	+	o
"	+	.002 cc.	+	o
"	+	.001 cc.	+	o

From these experiments it becomes evident that the action of the goose corpuscles is practically the same, whether we use the unaltered red cell, the laked blood, the stroma, or even the washings from the stroma. In all cases a strong agglutination was produced, practically always in dilutions of one to one thousand and frequently in dilutions of one to one thousand five hundred, while complete agglutination for the normal serum, although relatively high, never exceeded a limit of about one to two hundred and fifty, a strength established by the examination of a large number of normal rabbits.

At no time was lysis present beyond the limits of normal variation. Several explanations could be afforded for this phenomenon. It might first be possible that we had succeeded in separating lysis from agglutination, producing only agglutination where goose corpuscles were used for injection. Again, it might be possible that sufficient complement was lacking in rabbit serum to effect the solution of the goose corpuscles, even though the immune body were present in the serum to unite with the erythrocytes and agglutinate them.

A series of observations was therefore made to determine whether other species of animals might furnish a complement in their serum for the agglutinated goose blood, or whether normal goose serum might not effect the same results if added in proper quantity. The sera from a number of animals, including normal goose, hen, pigeon, rabbit, guinea-pig, dog, swine, and man, were tested to determine the limit of dilution, at which no lysis or agglutination of goose erythrocytes occurs, and to establish a standard dilution, which might be used for adding to the agglutinated erythrocytes an excess of complement.

With the exception of dog's serum, which dissolved goose blood in high dilutions, none of these sera proved to have any marked action upon unaltered goose corpuscles. Mixtures of goose's blood in suspension and varying dilutions of rabbit's serum, obtained by the injection of goose's blood, were prepared and allowed to stand at 3° Centigrade for two hours. Other mixtures of inactivated serum and goose corpuscles were prepared and kept at room temperature for the same time. The goose corpuscles, now firmly agglutinated, were freed from serum by centrifugation, and the volume made up to one cubic centimeter, the original quantity, by the addition of normal salt solution. To each of these mixtures was now added serum from the various normal animals in the proper dilution and the preparations placed in the thermostat.

With the exception of the guinea-pig serum, none of the mixtures so made showed a trace of lysis. In other words, the substances necessary to effect the solution of the agglutinated goose corpuscles were not present in the serum from normal goose, rabbit, hen, pigeon, pig, or man. A trace of lysis was observed from the addition of guinea-pig serum, which is especially rich in complemental substances, and which, therefore, seemed suitable for the purpose in view. The use of a number of samples of guinea-pig's serum indilutions too low to effect any solution of normal goose erythrocytes demonstrated that in the serum of this animal[1]

[1] This complement was not found in the serum of all the guinea-pigs tested.

sufficient complement was present to act upon agglutinated goose erythrocytes and to dissolve them in solutions which we had previously established as the limit of agglutination. This result is shown in the following table.

TABLE IV.

Series D, I.

Rabbit treated with goose's corpuscles. Seven injections.

5% Suspenson Goose Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	Trace.
"	+	.01 cc.	+	0
"	+	.005 cc.	+	0
"	+	.001 cc.	+	0
"	+	.00067 cc.	+	0
"	+	.0005 cc.	0	0

Preparations placed on ice at 3° Centigrade for two hours, then centrifugalized, the agglutinated corpuscles washed, volume made up to one cubic centimeter by the addition of normal salt solution.

Series D, II.

Normal guinea-pig serum.

5% Suspension Goose Corpuscles.		Normal Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.5 cc.	+	+
"	+	.3 cc.	Trace	Trace.
"	+	.2 cc.	Trace	Trace.
"	+	.1 cc.	Negative	Negative.

Guinea-pig serum which in dilutions of one to ten is thus seen to be without action upon goose's blood was now added to the agglutinated goose corpuscles and the results obtained are given in the following protocol.

Series D, III.

5% Goose Corpuscles.		Immune Serum.		Normal Guinea-pig Serum.	Lysis.
1 cc.	+	1. cc.	+	.1 cc.	+
"	+	.1 cc.	+	"	+
"	+	.01 cc.	+	"	+
"	+	.005 cc.	+	"	+
"	+	.001 cc.	+	"	+
"	+	.00067 cc.	+	"	Trace.
"	+	.0005 cc.	+	"	Negative.

We thus see that by the addition of normal guinea-pig's serum in .1 cubic centimeter quantities, we have obtained a complete lysis in a dilution of 1-1000, the same dilution in which we found complete agglutination in our "immune serum." In short, by the injection of rabbit's with goose's corpuscles we have produced an immune body capable of uniting with and agglutinating goose erythrocytes in high dilutions. For the solution of these altered erythrocytes, however, the necessary complemental substances do not exist in the diluted rabbit's serum, but must be furnished in excess from the serum of normal guinea-pigs. When this serum is added, complete lysis is always brought about.

Similar experiments were made with the sera of rabbits which had received injections of laked goose's blood, stroma, and the second washing. In each case, the addition of .1 cubic centimeter of guinea-pig serum to the agglutinated erythrocytes was followed by solution of the cells.

It has not been deemed advisable to publish tables of these tests, as Table III., Series C, III., IV., and V., shows the

agglutinating power of these sera, and, in each case, the addition of guinea-pig serum in .1 cubic centimeter amounts was followed by lysis, where agglutination had taken place.

A series of similar observations was now conducted with the serum from the guinea-pigs treated with rabbit's blood, where a complete agglutination and a partial lysis had been observed previously. The results are given in the following tables.

TABLE V.

Series E, I.

Guinea-pig treated with rabbit's blood, laked. Six injections.

5% Suspended Rabbit's Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	Trace.
"	+	.05 cc.	+	0
"	+	.04 cc.		0
"	+	.03 cc.	+	0
"	+	.02 cc.	+	0
"	+	.01 cc.	Trace	0

Placed on ice at 3° Centigrade, after two hours agglutinated corpuscles centrifugated, washed free from serum; volume made up to 1 cubic centimeter by addition of normal salt solution.

Series D, V.

Normal guinea-pig serum.

5% Rabbit Corpuscles.		Normal Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.5 cc.	Trace	Trace.
"	+	.1 cc.	Negative	Negative.

Normal guinea-pig serum in .1 cubic centimeter doses was now added to the agglutinated rabbit's corpuscles with the following result:

Series D, VI.

5% Rabbit Corpuscles.		Immune Serum.		Normal Guinea-pig Serum.	Lysis.
1 cc.	+	1 cc.	+	.1 cc.	+
"	+	.1 cc.	+	"	+
"	+	.05 cc.	+	"	+
"	+	.04 cc.	+	"	+
"	+	.03 cc.	+	"	+
"	+	.02 cc.	+	"	+
"	+	.01 cc.	+	"	Partial.

We thus see that, whereas in the serum from guinea-pigs treated with laked blood of rabbits only an agglutination is present, a complete lysis is brought about by the addition of fresh guinea-pig serum (to furnish excess of complement), in which case lysis is present in dilutions of one to fifty, a partial solution of the corpuscles occurring in a dilution of one to one hundred. A similar result was brought about in the serum resulting from the injection of the rabbit's stroma.

Series D, VII.

Guinea-pig treated with rabbit stroma. Six injections.

5% Rabbit Corpuscles.		Immune Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	+	Trace.
"	+	.05 cc.	+	0
"	+	.04 cc.	+	0
"	+	.03 cc.	+	0
"	+	.02 cc.	+	0
"	+	.01 cc.	Trace	0

Placed on ice at 3° Centigrade for two hours, then centrifugalized and washed. Volume made up to 1 cubic centimeter by the addition of normal salt solution.

Normal guinea-pig serum in dilutions of one to ten was now added to these agglutinated corpuscles, with the following result:

Series D, VIII.

5% Rabbit Corpuscles.		Immune Serum.		Normal Guinea-pig Serum.	Lysis.
1 cc.	+	1 cc.	+	.1 cc.	+
"	+	.01 cc.	+	"	+
"	+	.05 cc.	+	"	+
"	+	.04 cc.	+	"	+
"	+	.03 cc.	+	"	+
"	+	.02 cc.	+	"	+
"	+	.01 cc.	+	"	Trace.

Thus in the serum from animals treated with rabbit's stroma, as in the case of the laked blood, we find a complete lysis, demonstrable by the addition of complement from normal guinea-pig serum, occurring in the same dilutions in which the aggutinations without lysis occurred in our original immune serum.

In brief, in all samples of sera obtained by the injection of the entire red cell, or the various constituents of this cell, in which we find, in the higher dilutions, merely agglutination, by the addition of sera rich in complemental substances we are able to produce lysis, as well as agglutination, and to demonstrate that we are dealing with a veritable immune body.

HEMOGLOBIN EXPERIMENTS

The animals treated with dog's hemoglobin, prepared by the above described method, furnished sera which, even after repeated injections, differed from the normal in no particular. Both rabbits and guinea-pigs were repeatedly injected with

solutions of pure hemoglobin prepared from dog's blood, and the sera tested for lytic and agglutinating substances. In all cases the sera of these animals dissolved and agglutinated dog's erythrocytes only in the same dilutions as did the sera of normal animals.

As the results are negative, a protocol of but one experiment is given:

Series E, I.

Rabbit treated with dog's hemoglobin. Twelve doses (6–7 concentrated solution at each dose).

5% Dog's Corpuscles.		Rabbit Serum.	Agglutination.	Lysis.
1 cc.	+	1 cc.	+	+
"	+	.1 cc.	0	0

Control from normal rabbit same as above.

The tests for precipitins, carefully and repeatedly made, in all cases gave negative results. Portions of the sera from the rabbits and guinea-pigs which had received the injections were mixed with concentrated hemoglobin solutions in the proportion of one to one. Similar mixtures of normal sera and hemoglobin solution served as controls. The tubes were allowed to stand for twenty-four hours, and then examined. In no case was the precipitating action of the immune (?) serum greater than that of the normal used as a control. In some cases the normal serum showed a more powerful precipitin action than the serum of the animal which had received injections of hemoglobin.

The experiments, in which we endeavored to produce specific immune bodies by the injection of pure hemoglobin prepared from hen's blood, did not give uniform results. Two rabbits, treated with numerous injections of this hemoglobin, developed strong agglutinating and lytic properties in their sera. As the purity of the hemoglobin was not above suspicion, this series was repeated by one of us (H.) in

Montreal, where the climatic conditions render the preparation of this hemoglobin less difficult. Two rabbits which received repeated injections of hen's hemoglobin failed to develop specific immune bodies. The onset of warm weather prevented further investigation, and we must for the present leave this question unsettled.

In none of these rabbits injected with hen's hemoglobin could specific precipitins be demonstrated.

From the various experiments detailed above, which we have duplicated in every case,[1] the protocols of but the more important experiments being included in this paper, we feel justified in offering the following general conclusions:

I. The employment of the constituents of the blood corpuscles of one species of animal, laked blood and stroma, for the injection of other species of animals results in the production of definite specific bodies — lysin and agglutinins.

II. In a strongly hemolytic serum the rapid solution of the corpuscles masks the appearance of the agglutination, which may be demonstrated in preparations kept on ice at 3° Centigrade, or by the use of inactivated serum.

III. In an immune serum, capable of uniting in high dilutions with the erythrocytes originally employed, the lysis in these dilutions is frequently absent, even though agglutination takes place, owing to the lack of sufficient complement in the diluted serum. The addition of excess of complement, in the shape of fresh normal serum, always avails to cause the solution of the corpuscles in the same dilutions in which they are agglutinated.

IV. Bordet's view that the stroma is responsible for the lysis and Nolf's view that the stroma is responsible for the agglutination and the laked blood for the lysis are both confirmed by the demonstration of both agglutination and lysis from the injection of both laked blood and stroma.

V. Contrary to van Dungern's view, the splitting up of the blood corpuscles by the use of distilled water does not result in the destruction of the substances in the corpuscle producing lysis and agglutination.

[1] In many of the series four animals were used.

VI. Finally, the phenomena of agglutination and lysis cannot be separated from each other by the injection of the constituents of the blood corpuscle, but these phenomena seem to be inseparably connected.

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