ON THE ANTIGENS OF RED BLOOD CORPUSCLES.

II. Flocculation Reactions with Alcoholic Extracts of Erythrocytes.*

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In a previous paper[1] it was pointed out that not only the heterogenetic substance present in sheep blood (Sordelli and Pico, Sachs and Guth), but alcoholic extracts of erythrocytes in general give flocculation reactions with hemolytic immune sera.

A further study of these reactions was undertaken in the following experiments. It was stated previously that the strongest reactions were obtained with immune sera resulting from injections of alcoholic extracts of blood (mixed with serum), but that they also occurred with common anti-eryhrocyte sera. The first object was to obtain further information regarding the specificity of these reactions.

EXPERIMENTAL.

A rather large number of different hemolytic immune sera, which were at our disposal, were tested with emulsions of alcoholic extracts of various bloods (Table I).

The extracts and emulsions were prepared as follows: 20 cc. of oxalated blood was centrifuged, washed 3 times with saline solution, and to the sediment 50 cc. of 95 per cent alcohol was slowly added, while shaking, to prevent the formation of clumps. After standing for 24 hours at room temperature the alcohol solution was filtered, and the residue was again extracted in the same way with 20 cc. of 95 per cent alcohol. The combined alcoholic extracts were filtered and concentrated to 10 cc., corresponding to one-half the original blood volume. After cooling to room temperature this solution was filtered again to remove any turbidity. The term "alcoholic extract" used in this communication refers to this kind of solution, unless special mention is made of a change in technique.

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* Twenty-first paper on antigens and specificity.

[1] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1925, xli, 427; *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 170.

The emulsions used for the flocculation reactions were prepared in different ways. The original method employed by us, of adding 5 parts of saline, drop by drop, to 1 part of alcoholic extract (method of Sachs and Guth for heterogenetic antigen) could not be used equally well for all kinds of blood. In some cases the emulsions were not stable enough, and for this reason yielded too many weak heterologous reactions, undesirable for the study of the specificity. It was found possible to increase the stability of the emulsions by a quicker addition of the saline solution, and the most stable emulsions were those obtained by quickly adding the alcoholic extract to the salt solution (*cf.* Sachs). For each kind of blood the most suitable method of emulsification had to be determined by preliminary experiments. It even happened occasionally that extracts of bloods of the same species, and apparently made in the same way, showed differences in the stability of their emulsions. In the case of rat blood it was found necessary to use the acetone-insoluble fraction of the extract and to add cholesterol. After evaporating the alcoholic extracts to dryness, and redissolving the residue with boiling absolute alcohol, extracts were obtained which still reacted typically. Furthermore, filtration of the solution in absolute alcohol through Berkefeld filters did not remove the active substances. For each extract the tests with all the sera were made on the same day and with the same freshly prepared emulsion.

Preparation of the Emulsions Used in the Experiment of Table I.

1. Horse Blood.—To 1 part of alcoholic extract of blood 5 parts of saline were added, drop by drop while shaking.

2. Chicken Blood.—Prepared in the same manner, with addition of saline very slowly, drop by drop.

3. Rat Blood.—10 cc. of alcoholic extract, corresponding to 20 cc. of blood, was evaporated to dryness and extracted twice with 20 cc. of boiling acetone. The residue was extracted with 70 cc. of 95 per cent alcohol on the steam bath, the alcoholic solution filterd hot, concentrated to 10 cc., and again filtered after cooling. To 5 cc. of this alcoholic solution was added 0.3 cc. of a 1 per cent alcoholic cholesterol solution, in alcohol, and then 5 times the volume of saline, drop by drop. (Without cholesterol only weak reactions were obtained. The original alcoholic extract gave emulsions flocculating by themselves.)

4. Human Blood.—1 part of alcoholic extract was blown from a pipette into 5 parts of saline.

5. Rhesus Blood.—In the same way as 4.

6. Pig Blood.— " " " " " "

7. Sheep Blood.— " " " " " "

8. Horse Kidney.—1 part of minced horse kidney was extracted with 5 times the volume of 95 per cent alcohol for 48 hours, at room temperature. The alcoholic solution was filtered, and 1 part was blown into 5 parts of saline.

9. Guinea Pig Blood.—No entirely satisfactory method has been found for this blood extract. The alcoholic extract was diluted 5 times with 95 per cent

alcohol, and 1 part was blown into 5 parts of saline. In practically every tube, including the control, slight flocculations occurred. The table gives only the readings of the distinct reactions.

To 0.2 cc. of the inactivated rabbit immune sera, diluted one-half, 0.2 cc. of a given emulsion was added, and the tubes were kept at 37°C. for 20 hours.

In the readings both the formation of flakes and of sediment were taken into account. The strength of the reactions is indicated as follows: − = negative; F.tr. = faint trace; Tr. = trace; ±, +, +±, etc.; ? = questionable; blank spaces = no test made. Several times we noticed that the reactions had increased in intensity when the tubes were allowed to stand longer than the usual period of 20 hours. An example is given in the last column of the table (pig blood).

One sees from the experiments that a considerable number of the immune sera react distinctly on the emulsions of homologous blood extract, but to different degrees. In the case of the immune sera against human blood of Group I and horse blood, the reactions were comparatively weak. The differences in the effect of the individual immune sera are certainly due in part to their unequal hemolytic or agglutinative strength; but the existence of qualitative differences has also to be considered.[1]

Besides the homologous reactions others occurred. If one disregards the faint ones, these heterologous reactions display a certain regularity. An example is to be found in the frequency of reactions of horse, donkey, and mule blood antisera with emulsions of alcoholic extract of rat blood. Six out of ten immune sera for horse blood, five for donkey blood, and three out of ten for mule blood gave flocculations with rat blood extract, most of them of considerable strength, while none of the other heterologous sera reacted. That these reactions were mostly stronger than those on horse blood extract may be explained by the fact that cholesterol was used in the preparation of the rat blood extract emulsion, and not for the horse blood extract. There was no visible effect of the rat blood immune sera on horse blood extract. A similar case is to be found in the action of rhesus and baboon blood immune sera on pig blood extract. Moreover, it will be noted that the two immune sera against monkey blood also flocculate human blood extracts, while the reverse reaction is rare and faint. Another group of such reactions is that of the blood corpuscles known to be related to the so called "Forssman antigen;" *viz.*,

TABLE I.

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of										
		Human blood Group I.	Human blood Group II.	Sheep blood.	Horse kidney.	Chicken blood.	Horse blood.	Rat blood.	Guinea pig blood.	Rhesus blood.	Pig blood.	Pig blood.*
Human blood Group I.	17	Tr.	F.tr.	−		−	−	−	−			
	18	−	−	−	−	−	−	−	−	−	−	−
	19	−	−	−	−	−	−	−	−	−	−	−
	23	−	−	−	−	Tr.	−	−	−	−	−	+±
	24	−	−	−	−	−	−	−	−	−	−	−
	25	+±	Tr.	−	−	−	−	−	−	Tr.?	F.tr.	+
	26	−	−	−	−	−	−	−	−	−	−	−
	27	Tr.	Tr.?	−	−	−	−	−	−	−	−	−
	64	−	−	F.tr.	±	−	−	−	−	−	−	±
	65	−	−	−	−	−	−	−	−	−	−	Tr.
	4–63	−	F.tr.	−	−	−	−	−	−	−	−	−
	4–64	±	Tr.	−	−	−	−	−	−	F.tr.	−	−
Human blood Group II.	20	−	+±	+	−	−	−	−	−	−	−	−
	21	+	+++	+±	−	−	−	−	−	Tr.	−	+±
	22	±	+++	++±	−	−	−	−	−	−	−	+
	3–43	−	±	−	−	−	−	−	−	−	−	−
	3–44	−	F.tr.	Tr.	Tr.	−	−	−	−	−	−	−
	3–45	−	−	−	−	−	−	−	−	−	−	−
	3–46	−	F.tr.	F.tr.	−	Tr.	−	−	−	−	−	−
	4–14	−	++±	++	−	−	−	−	−	−	−	−
	4–15	−	F.tr.	F.tr.	−	−	−	−	−	−	−	−
	4–16	−	−	−	F.tr.	−	−	−	−	−	−	−
	4–17	−	−	F.tr.	−	−	−	−	−	−	−	−
	4–18	+	Tr.	−	−	−	−	−	−	−	−	F.tr.
	4–19	±	+	Tr.	−	±	−	−	−	−	−	+±

Rhesus blood.	4-56	++	+±	−	−	−	−	−	−	+±	Tr.	++
	4-67			−	−					++±	+±	++±
Baboon blood.	4-58	+	+±	Tr.	−	−	−	−	−	+±	+	++±
	4-59			−	−						Tr.	++
Sheep blood.	10	−	−	+±	+	−	−	−	Tr.			
	11	F.tr.	+++	+++	+++	++	−	−	F.tr.			
	12	F.tr.	−	++	++	Tr.	−	−	Tr.	Tr.?	−	F.tr.
Horse kidney.	54	−	+	+++	++±	++	F.tr.	−	−	−	−	Tr.
	4-02	−	−	+++	++±	++	F.tr.	−	+	Tr.	−	+
	4-03	−	−	+++	+	++	−	−	−	−	−	−
	4-04	−	−	+++	±	++	−	−	−	−	−	−
Beef blood.	4-32	−	−	−	−	−	−	−	−	−	−	−
Horse.	1	Tr.	−	−	−	−	±	+++	−			
	3	−	−	−	−	−	±	−	−	−	−	−
	30	−	−	−	−	−	Tr.	++	−	−	−	−
	31	−	−	−	−	−	Tr.?	−	−	−	−	−
	32	−	−	F.tr.	F.tr.	−	+	±	−	Tr.?	−	F.tr.
	33	−	F.tr.	−	−	−	−	++	−	−	−	+
	34	−	−	−	−	−	+	+++	−	−	−	−
	35	−	−	F.tr.	−	−	Tr.	+++	−	−	−	−
	2-62	−	−	−	−	−	Tr.	−	−	−	−	−
	3-03	−	−	−	−	−	−	−	−	−	−	−
Donkey blood.	36	−	−	−	−	−	±	+++	−	−	−	−
	37	−	−	−	−	−	++	+++	±	−	−	−
	38	−	−	−	−	−	−	+	−	−	−	−
	44	−	−	−	−	−	−	++	−	−	−	−
	2-74	−	−	−	−	−	Tr.?	F.tr.	−	−	−	−

* Same tests as in the preceding column after standing 1 more day at room temperature.

TABLE I—*Concluded.*

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of										
		Human blood Group I.	Human blood Group II.	Sheep blood.	Horse kidney.	Chicken blood.	Horse blood.	Rat blood.	Guinea pig blood.	Rhesus blood.	Pig blood.	Pig blood.*
Mule blood.	60	–	–	–	–	–	–	–	–	–	–	–
	61	–	–	–	–	–	–	–	–	–	–	–
	74	–	–	–	–	–	–	–	–	–	–	–
	76	–	–	–	–	–	–	–	–	–	–	–
	3-05	–	–	–	–	–	–	–	–	F.tr.	–	–
	3-07	–	–	–	–	–	–	–	–	–	–	–
	3-08	–	–	–	–	–	F.tr.	–	–	–	–	–
	3-32	–	–	F.tr.	Tr.	–	Tr.	+±	–	–	–	–
	3-34	–	–	–	–	–	++	++	–	–	–	Tr.
	3-39	–	–	–	–	–	++	++	–	–	–	–
Rat blood.	29	–	–	F.tr.	–	–	–	+++	+	–	–	F.tr.
	66	–	–	–	–	–	–	–	–	–	–	–
	67	–	F.tr.	F.tr.	Tr.	–	–	+++	–	–	–	–
	77	–	–	–	–	–	–	–	–	–	–	–
	79	–	–	–	–	–	–	–	–	Tr.?	–	–
	80	–	–	–	–	–	–	–	–	–	–	Tr.
	86							++				
	88	–	–	–	–	–	–	±	–	–	–	–
	96	–	–	–	–	–	–	–	–	–	–	–
Guinea pig blood.	8	–	–	–	–	–	–	–	++	–	–	–
	21	–	F.tr.	–	–	–	–	–	++	–	–	–
	68	–	–	–	–	–	–	–	++	–	–	–

Chicken blood.	41	–	–	+++	±	++±	F.tr.	–	–	–	–	–
	43	–	–	+++	F.tr.	±	–	–	–	–	–	–
	3–64	–	–	+++	±	+±	–	–	–	–	–	–
Duck blood.	3–58	–	Tr.	Tr.	F.tr.	Tr.	Tr.	–	Tr.	–	–	Tr.
	3–59	–	Tr.	Tr.	Tr.	–	–	–	–	–	–	–
Human serum.	2	–	F.tr.	–	–	–	–	–	–	–	–	–
Horse serum.	2–95			–	–					–	–	Turbid.
	2–99	–	–		–	–	–	–	–			
Pig serum.	3–85	Tr.	±	–	–	Tr.	–	–	–	Tr.	–	+±
Rat serum.	83	–	–	–	–	–	–	±	–	–	–	–
Guinea pig serum.	2–03	–	–	–	–	–	–	–	±	–	–	–
Chicken serum.	50	–	–	++	–	+±	–	–	–	–	–	±
Normal rabbit sera.	4–90	–	–			–	–	–	–			
	4–91	–	–			Tr.	–	–	–			
	4–92	–	–			–	–	–	–			
	4–93	–	–			+	–	–	–			
	4–94	–	–			–	–	–	–			
	5–16			–	–					–	–	–
	5–17			–	–					–	–	–
	5–19			–	–					–	–	±
	5–24			–	–					–	–	–
	5–28									–	–	–

sheep, chicken, Group II human blood. To this group also belong the flocculations with alcoholic extract of horse kidney.

The weak reactions which take place in a rather irregular way may be due to the presence of the same or similar substances in all the extracts, or to the action of certain antibodies on various substances.

In tests with normal rabbit sera only two weak and one moderate reaction occurred, the latter in the case of chicken blood extract. The reacting serum was the only one of the five normal sera tested which gave considerable hemolysis with chicken blood. When normal sera of several other animals and human sera were allowed to act on various blood extracts, the readings being taken after 1 day at 37°C., the tests were mostly negative, except in the case of the horse blood extract. This was flocculated by normal chicken, sheep, and beef sera, the latter reactions being strong. The reaction became weaker, but did not entirely disappear when the beef serum was treated several times with horse blood. After standing 1 more day at room temperature, some other reactions became evident, for instance a strong flocculation of chicken blood extract by beef serum. A flocculation of a methyl alcohol extract of rabbit blood with chicken serum has already been described by Bordet.[2] Pick and Schwarz[3] reported the flocculation of extract of beef serum by normal rabbit serum, and of horse serum extract by the serum of a rabbit injected with this material.

Table II gives the flocculation reactions of a special group of immune sera obtained by injection of a mixture of an alcohol or ether extract of horse blood with protein (diluted pig serum). The preparation of such sera and their properties were described in earlier communications.[1] As stated therein, these sera proved to be specific in their hemolytic action. In the flocculation tests, however, reactions on alcoholic extracts of several bloods were found. It is possible that this lack of specificity was caused by the injection of pig serum. However, no experiments were performed to decide this question.

The two sera prepared with ether extracts of blood alone flocculated only horse blood extract.

[2] Bordet, J., Studies in immunity, New York and London, 1909, 525.

[3] Pick, E. P., and Schwarz, O., *Biochem. Z.*, 1909, xv, 453.

TABLE II.

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of										
		Human blood Group I.	Human blood Group II.	Sheep blood.	Horse kidney.	Chicken blood.	Horse blood.	Rat blood.	Guinea pig blood.	Rhesus blood.	Pig blood.	Pig blood.*
Alcoholic extract of horse blood + pig serum.	15	±	+	−	−	F.tr.	++	−	−	+	±	+±
	16	−	−	−	−	−	+±	−	−	−	−	Tr.
	20	++	+±	Tr.	F.tr.	++	+±	−	−	−	−	+
	40	−	F.tr.	F.tr.	Tr.	−	+±	−	−	−	−	−
Ether extract of horse blood + pig serum.	4–23	−	−	−	−	±	Tr.	−	−	−	−	−
	4–26	−	−	F.tr.	−	−	Tr.	−	−	−	−	−
	4–28	++	+±	Tr.	−	++±	+++	−	−	Tr.	+	+±
	4–30	−	−	−	−	Tr.	±	−	−	−	−	−
Ether extract of horse blood.	4–38	−	−			−	+±	−	−			
	4–62	−	−			−	++	−	−			

A small number of precipitin sera were tested. Some of them reacted, in most cases on the extract of the homologous blood; but the majority of the reactions were weak (Table I).

According to former results, the property of an immune serum to flocculate seems to run parallel with its content in hemolysins rather than that of agglutinins.[1] In order to see whether the antibodies concerned in the hemolytic and flocculating reactions are identical, we removed the hemolysins and agglutinins in the usual way, by addition of erythrocytes (Table III).

TABLE III.

Immune sera prepared by injection of	Immune serum No.	Absorbed with	Emulsion of extract of horse blood.
Horse blood.	32		+±
" "	32	Horse blood.	−
" "	34		++
" "	34	Horse blood.	+
Alcoholic extract of horse blood + pig serum.	15		++±
" " "	15	Horse blood.	++
" " "	20		++±
" " "	20	Horse blood.	−

The absorption was made by repeated addition of washed blood sediment to the one-half diluted inactivated immune serum (for example, 0.35 cc. of sediment to 0.5 cc. of immune serum in 6 portions, allowing the mixture to stand in the ice box for 1 hour each time until finally no hemolysins or agglutinins could be detected in the super-fluid). The flocculation tests were made as before.

The results suggest that in certain sera a part only of the antibodies producing flocculation are identical with the hemolysins. This view is substantiated by experiments to be described.

Group-Specific Flocculations with Alcoholic Extracts of Human Blood.[4]

It is known that immune sera prepared with human blood of a certain group may contain group-specific agglutinins.[5] As shown in

[4] In cooperation with Dr. Dan H. Witt.

[5] Landsteiner, K., *Wien. klin. Rundschau*, 1902, No. 40. Hooker, S. B., and Anderson, L. M., *J. Immunol.*, 1921, vi, 419.

a preliminary communication[6] such immune sera for blood II, if sufficiently strong, produce a heavy flocculation with extract of blood of Group II, and act much less strongly upon extracts from Groups I and III. In these comparative experiments the emulsions were prepared by strictly identical technique (Table IV).

It appears from these results that group-specific substances can be extracted from human erythrocytes by means of 95 per cent alcohol. Also absolute alcohol can be used with the same result.

In the paper referred to, no mention was made of the immune sera against Group III blood. With the use of the usual technique, the results were not very distinct, but good flocculations were obtained if the tests were allowed to stand at room temperature for 2 days. However, under these conditions, the flocculations produced by immune serum II with extracts Group II were weaker than usual.

According to these tests (Table IV, *a*), Group III immune sera have the strongest effect upon Group III extracts. A comparison of the phenomena just described with the results of Schiff and Adelsberger[7] will be given below.

Heterologous (Heterogenetic) Reactions.

Monkey Blood Immune Sera-Pig Blood.—In view of the effects, already mentioned, of monkey immune sera on pig blood extract, we made hemolytic and agglutinin tests with these sera on pig blood. The titrations were made as described[1] (Table V).

Fifteen other hemolytic immune sera, including anti-human immune serum, and five normal rabbit sera did not effect complete hemolysis of pig blood in a dilution of 1/50, and most of them were not at all, or only faintly hemolytic. The difference between the monkey blood immune serum and the other immune sera was not pronounced in the agglutinin tests. As the experiments show, the heterogenetic reaction of monkey blood immune serum on pig blood

[6] Landsteiner, K., van der Scheer, J., and Witt, D. H., *Proc. Soc. Exp. Biol.* 1924–25, xxii, 289.

[7] Schiff, F., and Adelsberger, L., *Z. Immunitätsforsch., Orig.*, 1924, xl, 335; *Klin. Woch.*, 1924, iii, 679; *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, 172. See Hesser, S., *Acta med. Scand.*, 1924, suppl. 9.

TABLE IV.

Read after 20 hours at 37°.

Immune sera prepared by injection of	Immune serum No.	0.2 cc. emulsion of alcoholic extracts of corpuscles.									
		Blood 1 Group I.	Blood 2 Group I.	Blood 3 Group I.	Blood 4 Group I.	Blood 5 Group II.	Blood 6 Group II.	Blood 7 Group II.	Blood 8 Group II.	Blood 9 Group III.	Blood 10 Group III.
Human blood Group II....	21	F.tr.	F.tr.	Tr.	+	+++	+++	+++	+++	F.tr.	0
" " " II....	22	F.tr.	F.tr.	Tr.	+	+++	+++	+++	+++	0	0

TABLE IV*a*.

Read after 2 days at room temperature.

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of					
		Blood 11 Group I.	Blood 12 Group I.	Blood 5 Group II.	Blood 14 Group II.	Blood 15 Group III.	Blood 16 Group III.
Human blood Group III.....	4-78	+	+±	+±	+±	++±	++±
" " " III.....	4-80	−	−	Tr.	Tr.	+±	±
" " " III	4-81	+	+	+	+	+++	++±

can be demonstrated also by the hemolytic test. The hemolytic titer of the immune sera was rather low, but two of the three baboon blood immune sera had a higher titer for pig blood than for the homologous blood. This heterogenetic reaction is in no way connected with Forssman's heterogenetic antigen, as the immune sera under discussion do not flocculate distinctly extracts of sheep blood or of horse kidney. They have no marked hemolytic action on sheep blood.

Horse Blood Immune Sera-Rat Blood.—A second instance of a heterogenetic reaction brought to light by the flocculation method was

TABLE V.

Bloods.	Titer of agglutinin.			Complete hemolysis in dilution up to			Flocculations with emulsions of alcoholic extracts of	
	Rhesus.	Baboon.	Pig.	Rhesus.	Baboon.	Pig.	Rhesus.	Pig.
Rhesus blood I.S. 4–56.....	800		100	200		50	+±	Tr.
" " " 4–67.....	800		100	200		50	++±	+±
Baboon " " 4–58.....		800	200		<50	100	+±	+
" " " 4–59.....		600	200		50	200		Tr.

TABLE VI.

	Emulsion of extract of rat blood.	Emulsion of extract of horse blood.
Horse blood I.S. 34.....	+++	+±
Same after absorption with rat blood.....	+±	+
" " " " rabbit "	+++	

that of horse blood immune sera on rat blood extract. It was not demonstrable by hemolytic and agglutinin tests. One may infer that an immune serum, acting on a certain substance contained in erythrocytes, need not necessarily have the property of producing hemolysis or agglutination. On the other hand, some observations indicate that if a cellular antigen is divided into fractions, the total of the latter is not so active as the original material. If, for example, blood corpuscles are extracted with alcohol, there is a considerable loss in antigenic and binding properties.

Despite the lack of any visible action on the rat corpuscles, a part of the flocculating antibodies can be absorbed by rat blood (Table VI).

Heterogenetic Antigens of the Forssman Type.—According to the observations of Forssman and other workers along the same lines, a certain type of antigen is present in sheep blood and in cells of very different derivation; as, for example, in those from organs of the horse, guinea pig, etc. Chicken blood corpuscles belong to this group also (Kritschewsky,[8] Hyde[9]). Moreover, Schiff and Adelsberger[7] have described a relationship between human corpuscles Group II and the Forssman antigen.

The tests presented in Table VII give a picture of the relationship of various antigens of the Forssman type. They show that there are certain differences between these various substances.

From a few preliminary experiments, it would seem that dog blood may contain a substance related to the Forssman antigen. The same applies to horse serum, since we have observed reactions of immune sera against sheep blood, chicken blood, and horse kidney on alcoholic extracts of horse serum considerably stronger than those produced by other immune sera.[10]

Differences between the Antigen of Human Blood Group II and the Forssman Antigen.—According to Schiff and Adelsberger, some Forssman immune sera contain an agglutinin against human blood of Group II, and some immune sera prepared with human Group II blood contain hemolysins for sheep blood. This lysin for sheep blood can be absorbed by human corpuscles of Group II, while the sheep lysin of Forssman immune sera is not absorbed by human blood of Group II. We were able to confirm these results. For the above reason the two kinds of antigen are supposed by Schiff and Adelsberger not to be identical. These authors did not obtain distinct flocculation of human blood extracts with human Group II immune sera, but the same sera gave flocculations with alcoholic extracts of Forssman antigen.

It is evident from our findings that the properties of the extracts of human blood Group II are not identical with the reacting substances

[8] Kritchewsky, I. L., *J. Exp. Med.*, 1916, xxiv, 233.

[9] Hyde, R. R., *Am. J. Hyg.*, 1925, v, 217.

[10] See Hanganutziu, M., *Compt. rend. Soc. biol.*, 1924, xci, 1457.

contained in extracts of horse kidney and chicken blood. Furthermore, the reactions of Group II substance and sheep blood extract do not run parallel (Table VII).

When our emulsions were prepared according to the technique of the Sachs-Georgi reaction (without the use of cholesterol) we found positive reactions of human Group II immune sera on extracts of Forssman antigen (horse kidney), but they were distinctly weaker than those with human Group II extracts. The difference between the

TABLE VII.

In this table some results of Table I and additional tests are summarized.

Immune sera prepared by injection of	Immune serum No.	Emulsions of alcoholic extracts of				
		Human blood Group II.	Horse kidney.	Sheep blood.	Chicken blood.	Dog blood.
Human blood Group II.....	20	+±	−	+	−	Tr.
" " " II.....	21	+++	−	+±	−	F.tr.
" " " II.....	22	+++	−	++±	−	F.tr.
Horse kidney.....	54	+	++±	+++	++	++
" " *.....	4-02	−	++±	+++	++	
" "	4-04	−	±	+++	++	±
Sheep blood.....	11	+++	+++	+++	++	
" "	4-97	−	++±	+++	+±	+±
" "	4-99	±	++±	+++	++	++
Chicken "	41	−	±	+++	++±	+±
" "	43	−	F.tr.	+++	±	F.tr.
" "	3-64	−	±	+++	+±	F.tr.

* In connection with a paper by Trou-Hia-Hsü (Trou-Hia-Hsü, *Z. Immunitätsforsch., Orig.*, 1922, xxxiv, 507) it should be mentioned that some of the horse kidney immune sera, for instance No. 4-02, agglutinated sheep blood very distinctly.

Forssman extracts and those of human blood of Group II was still more striking with emulsions prepared in the manner described above. Under these circumstances, the human immune sera did not react on the extract of horse kidney (see Table VII).

In order to study the interrelation between the antigens in question, absorption experiments were carried out. The results are recorded in Table VIII. The tests confirm the difference between the flocculable substances of human blood Group II and sheep blood.

TABLE VIII.

Immune sera prepared by injection of	Emulsions of alcoholic extracts of							Hemolysis in dilutions up to	
	Human blood Group I.	Human blood Group II.	Horse kidney.		Sheep blood.		Chicken blood.		
	2	2	2	1	2	1	1	Sheep blood.	Chicken blood.
Human blood I.S. No. 21	−	+++			+	+++			
" " " " 21 after absorption with sheep blood	−	+±			−	F.tr.			
Human blood I.S. No. 22	−	+++			++	+++			
" " " " 22 after absorption with sheep blood	−	++			−	−			
Sheep blood I.S. No. 4–99			++±	+++	+++	+++	++±	1,600	400
" " " " 4–99 after absorption with human blood Group II			±	+±	+++	+++			
Sheep blood I.S. No. 4–99 after absorption with chicken blood			−	−	−	+±	−	400	<20
Sheep blood I.S. No. 4–99 after absorption with sheep blood			−	−	−	−		<20	

1 = prepared by adding 5 parts of saline, in drops, to 1 part of alcoholic extract.

2 = prepared by blowing 1 part of the alcoholic extract into 5 parts of saline. This emulsion is more stable than 1. The hemolytic tests were made as above, with 1 drop of 50 per cent blood.

DISCUSSION.

In preceding papers,[1, 11] the concept was set forth that the antigens of red cells consist of proteins which are combined with substances of a different chemical nature[12] not in themselves antigenic, or only weakly so, once they become separated from the complex antigen.

The specificity of proteins in general follows the arrangement of the zoological system, as was shown by the comprehensive experiments of Nuttall on serum proteins, and by the important investigations on hemoglobin, by Reichert and Brown. These authors were able to demonstrate that the relationship of the crystal form of hemoglobin parallels to a large extent the zoological relationship of the animals from which the hemoglobin is prepared. The antigens responsible for lysin and agglutinin reactions behave in a different way in that they display sharp differences in related, or even in the same species, and similarities in distant ones. Thus they are not in close correlation with the zoological scale. Examples supporting this view have been presented previously[11] (substances differentiating individuals of one species, Forssman's antigen, the results of absorption of normal and immune sera with blood of closely related species, the results of the isolation of agglutinin fractions from normal serum[13]). It should be emphasized again that after absorbing a normal serum with some kind of blood corpuscles and separating the absorbed agglutinins from the cells, the resulting agglutinin solutions regularly react upon red cells of various species, thus indicating the widespread existence of heterogenetic antigens.[13] Another fact to be mentioned in this connection, is the presence of factors similar to the human isoagglutinogens A and B in animals (von Dungern and Hirschfeld;[14]

[11] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1924, xl, 91; Landsteiner, K., and Simms, S., 1923, xxxviii, 127.

[12] In experiments performed with Dr. P. A. Levene, very active preparations of the binding substance (haptene) of Forssman's antigen, made from horse kidney, were found to give no biuret reaction.

[13] Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905; *Z. Hyg. u. Infectionskrankh.*, 1907–08, lviii, 213; Oppenheimer, C., Handbuch der Biochemie, Jena, 1910, ii, pt. 1, 410.

[14] von Dungern and Hirschfeld, *Z. Immunitätsforsch., Orig.*, 1910–11, viii, 526.

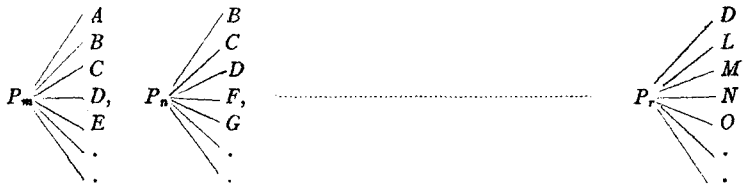
Landsteiner and Miller[15]).[16]

Our present work furnishes new instances of the existence of similar substances in blood of distantly related species, which can be extracted from the blood by alcohol. The flocculation method has proved of value for the detection of new cases of so called heterogenetic antigens. In one of them the relationship could be shown only by the flocculation reactions; in a second one it was also made evident by hemolytic tests. In view of all these observations the occurrence of so called heterogenetic antigens must be regarded as the rule rather than as the exception.

The assumption that there are present in erythrocytes of different species substances which are similar or identical would seem not to be in keeping with the fact that in most cases immune sera against erythrocytes are specific for the species. Yet it has been shown that an immune serum may not be markedly hemolytic for a certain blood and still contain an antibody active upon a fraction thereof, as in the case described of the flocculation of horse immune sera on rat blood extracts. Furthermore, reactions with high concentrations of immune serum are usually not taken into account, and erythrocytes of only a limited number of species have thus far been examined.

Since the whole corpuscles are much more apt to incite antibody production than are extracts, the proteins of the stromata must play an important part in the process of immunization. Consequently it is rather probable that the specificity of the anti-erythrocyte sera is influenced by the species-specific protein fraction.[17]

In the attempt to express the foregoing concept one may symbolize the serological structure of erythrocytes (and presumably of other animal cells) somewhat as follows:



[15] Landsteiner, K., and Miller, C. P., Jr., *Science*, 1925, lxi, 492.

[16] Chicken immune sera against human blood of Group III contain strong agglutinins for the blood of several animals, as those of the dog, guinea pig, pig, etc. (experiments made in cooperation with Dr. C. P. Miller, Jr., to be published later).

[17] See Avery, O. T., and Heidelberger, M., *J. Exp. Med.*, 1923, xxxviii, 73, 81.

A, B, C, etc., represent chemical substances combined with the protein (P) or groups contained in such substances. The symbols P_m and P_n indicate proteins that are nearly identical chemically and serologically. P_r is a dissimilar protein of a distant species. According to such a scheme most of the factors (A, B, etc.) are the same in closely related species (m, n), only a few being dissimilar, while in general the factors of distant species (m, r) are different, although identical ones may occur. This view is supported by the fact that when agglutinin immune sera are absorbed with heterologous blood more of the active substances are taken out by cells of a kindred species than by those of others. Also the individual differences within one species can be accounted for by variations of the factors A, B, etc. An idea which may be alternative or supplementary to the first is suggested by the fact that an antibody may react upon several substances not identical, but chemically similar.[18] Accordingly, the occurrence of a certain reaction need not necessarily imply the existence of a special serological factor in every case. Such a view would not require the assumption of a great number of substances in one kind of cells. Which of the two conceptions approaches the facts more closely can be a subject of further studies. The possibility that the reacting substances may be present in different amounts has also to be considered.

The meaning accorded to the specific factors, A, B, C, etc., differs from that embodied in the receptor theory in that the letters signify non-protein chemical substances (or groups of such a substance), separable from the complex antigen, and either not antigenic by themselves or only slightly so (haptenes). If the antigen is broken up, as by the action of alcohol, its antigenic power is greatly impaired. According to our conception, which is at variance with the current opinion, species specificity of cells is of a different order as opposed to species specificity of proteins.

SUMMARY.

1. Flocculation reactions of anti-erythrocyte sera on emulsions of alcoholic extract of blood are described.

[18] *Biochem. Z.*, 1918, lxxxvi, 343.

2. The reactions are markedly species-specific.

3. Besides the homologous reactions certain others—"heterogenetic" ones—have been observed, and in this way the existence of new examples of heterogenetic antibodies has been demonstrated.

4. Group-specific substances can be extracted from human erythrocytes with alcohol and demonstrated by flocculation with group-specific immune sera.

5. A conception of the structure of cellular antigens based upon the known facts, is presented.