PRODUCTION OF HETEROGENETIC ANTIBODIES WITH MIXTURES OF THE BINDING PART OF THE ANTIGEN AND PROTEIN.*

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In 1911 Forssman[1] discovered the fact that rabbits injected with suspensions of organs of guinea pigs and a few other animals produce antibodies capable of hemolyzing sheep blood. These hemolysins differ from those which are produced in the usual way by injection of sheep erythrocytes. Because of their origin, they were called heterogenetic antibodies. Later the corresponding antigens (heterogenetic antigens) were found to be present in the tissues of many kinds of animals, but absent in others. Some facts relating to the properties of heterogenetic antigens that have a direct connection with our investigation are the following:[2]

Doerr and Pick[3] detected the resistance of the antigen to alcohol, and Sachs and Georgi[4] made use of alcoholic extracts for their experiments. Friedberger[5] (with Poor, Suto, and Schiff) found the heterogenetic antigen present in the urine of animals to be soluble in alcohol (Doerr and Pick). Sordelli and his coworkers[6] pointed out that the extracts have not only, as known before, the property of combining with the corresponding antibodies but can be flocculated by the immune sera (*cf.* Sachs and Guth[7]). They stated that the extracts are devoid of any power to produce antibodies when injected into rabbits. On the other hand, these authors claimed that heterogenetic antibodies can be produced by injection of the alcohol-

* Sixteenth paper on antigens.

[1] Forssman, J., *Biochem. Z.*, 1911, xxxvii, 78.

[2] For a more complete quotation of the literature, consult the articles mentioned.

[3] Doerr, R., and Pick, R., *Biochem. Z.*, 1913, l, 129.

[4] Sachs, H., and Georgi, W., *Z. Immunitätsforsch., Orig.*, 1914, xxi, 346. *Cf.* Georgi, W., *Arb. k. Inst. exp. Therap. Frankf.*, 1919–20, No. 9, 43.

[5] Friedberger, E., and Suto, K., *Z. Immunitätsforsch., Orig.*, 1919, xxviii, 217.

[6] Sordelli, A., and coworkers, *Rev. inst. bact. Buenos Aires*, 1918, i.

[7] Sachs, H., and Guth, F., *Med. Klin.*, 1920, xvi, 157.

insoluble residue, in spite of its inactivity against the antibodies *in vitro*. In a later review[8] of their work the authors repeated this statement, adding that the antigenic activity of the residue is weak. Landsteiner[9] obtained similar results, but laid stress on the fact that the antigenic power of the residue after alcohol extraction is markedly diminished. Furthermore, it is completely, or almost completely destroyed by treating the material (horse kidney) for a short time with boiling alcohol.

One of the present authors has tried to explain the known facts by the assumption that the heterogenetic antigens consist of two parts, combined with each other and capable of being separated by the action of alcohol. One part is a protein and is necessary for the production of antibodies. The other, which is alcohol-soluble and perhaps a lipoid, contains the specific group but possesses no marked antigenic property and is active as an antigen when combined with proteins. This view was strengthened by experiments[10] which demonstrated that substances of a simple chemical constitution form antigens in combination with proteins and that the corresponding antibodies act specifically on these simple substances. Owing to the lack of a suitable term for such bodies, hitherto but not quite correctly spoken of as antigens, the term haptene was proposed in our previous communication. This term designates, therefore, substances which, while acting strongly and specifically with homologous antibodies, have little or no antigenic properties as compared with the binding power, but become antigens when combined with proteins. It is possible that there exist a number of such substances, as, for example, the alcohol-soluble products studied by K. Meyer[11] and Waelsch, and perhaps the bacterial substances described by Dochez and Avery,[12] Zinsser and Parker,[13] and Heidelberger and Avery.[14] It is still

[8] Sordelli, A., and Fischer, G., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 174.

[9] Landsteiner, K., *Proc. Acad. Sc. Amsterdam*, February, 1921, xxiii, 1166; *Biochem. Z.*, 1921, cxix, 294.

[10] Landsteiner, K., *Biochem. Z.*, 1919, xciii, 106; 1920, civ, 280.

[11] Meyer, K., *Z. Immunitätsforsch., Orig.*, 1910, vii; 1911, ix; xi; 1922, xxxiv.

[12] Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.

[13] Zinsser, H., *J. Exp. Med.*, 1921, xxxiv, 495. Zinsser, H., and Parker, J. T., *J. Exp. Med.*, 1923, xxxvii, 275.

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

questionable whether the so called Wassermann antigen has any relation to the present subject (see Seligmann[15]).

The foregoing hypothesis on the nature of the heterogenetic antigen was afterwards independently put forward by Taniguchi[16] and von Gutfeld[17] (see Doerr[18]). In view of the new facts and their interpretation, the existence of nearly related antigens in the tissues of non-related animals appears less surprising than it was previously thought. Such an occurrence would simply depend on the presence of one substance (or several similar substances) of comparatively simple constitution in all these tissues. With regard to the chemical nature of the haptene, according to K. Meyer,[19] it belongs to the group of lecithins and cephalins, while Wernicke and Sordelli[20] designate it as a cerebroside. We believe, however, that the facts put forward up to the present are not yet sufficient to warrant any definite statement as to the purity of the preparations or their precise chemical constitution, especially when one considers the intricacies of the chemical investigation of lipoids.

In order to test the hypothesis above mentioned, we tried to produce antigenic effects by combining the haptenes artificially with proteins—as it were, to synthesize the antigen. The result could not be predicted, as in our previous experiments new antigens were built up from chemical substances which could unite with proteins by means of definite chemical reactions. Such a reaction not being at our disposal in the present investigation, we tried to attain our object by simply mixing the alcoholic extracts with protein-containing solutions. It was thought that in this way a loose (adsorption) compound might be formed.[21] It may be recalled that the natural heterogenetic antigen is also, according to our opinion, a loose compound which can be split up by simple treatment with alcohol.[22]

[15] Seligmann, E., and Pinkus, F., *Z. Immunitätsforsch., Orig.*, 1910, v, 377.

[16] Taniguchi, T., *J. Path. and Bact.*, 1921, xxiv, 253, 254.

[17] von Gutfeld, F., *Z. Immunitätsforsch., Orig.*, 1922, xxxiv, 524.

[18] Doerr, R., *Ergebn. Hyg.*, 1922, v, 168, 130.

[19] Meyer, K., *Biochem. Z.*, 1921, cxxii, 225.

[20] Wernicke, R., and Sordelli, A., *Compt. rend. Soc. biol.*, 1921, No. 3; *Rev. inst. bact. Buenos Aires*, 1919, ii, 281.

[21] It is perhaps possible that the natural hemolysins which are present in the sera employed play a part in the formation of the supposed compounds.

[22] One of the experiments has been briefly reported previously (Landsteiner, K., *Biochem. Z.*, 1921, cxix, 306; *Proc. Acad. Sc. Amsterdam*, 1922, xxiv, 237).

EXPERIMENTAL.

For the injections rabbits were used. To obtain reliable results it was necessary to treat the different series of animals in the same way and at the same time and to use a sufficient number for each experiment. For although the action of heterogenetic antigen is remarkably constant (Doerr) the difference in the reaction of individual animals is great enough to mask the underlying principles when only a small number of animals are studied. Furthermore, in selecting the animals those were excluded in which the serum, prior to the injection, gave a strong or complete hemolysis in a dilution of 1:25 in half an hour under the conditions of the tests. In this way the initial hemolytic activity of the sera was rendered negligible. This method was considered better than to employ rabbits with hemolyzing sera and to calculate the ratio between the initial and the final values, because it is not known whether the production of antibodies in immunized animals is proportional to the initial amount of normal antibodies, other conditions being equal.

Preparation of the Material for Injection.—150 gm. of horse kidney were passed through a mincing machine, sometimes through a sieve. To the pulp were added 750 cc. of 95 per cent alcohol and the suspension was kept at room temperature for 2 days with occasional shaking. The suspension was then filtered and the insoluble residue similarly treated with 450 cc. of 95 per cent alcohol. The first extract was evaporated almost to dryness on a water bath, then dissolved in the filtrate of the second extraction by heat, filtered when hot, and evaporated nearly to dryness. The residue was emulsified in 50 cc. of 0.9 per cent saline.

From this stock solution the injection solutions were prepared by diluting twenty times with 0.9 per cent saline or in the same proportion with diluted serum. The saline emulsions and the mixtures with serum thus contained the same percentage of kidney extract. The sera (human or pig) were diluted eight to ten times with 0.9 per cent saline. To all solutions 0.25 per cent phenol was added. A sufficient quantity of the solutions was made for each series of injections and preserved in the ice box.

Preparation of Antibodies.—The rabbits, fed with vegetables and oats, were injected with 5 cc. of solution each time, intravenously or intraperitoneally, the injections being repeated five to six times at intervals of 7 to 10 days.

Tests for Hemolysis.—The sera of the rabbits were taken 7 days after the last injection. To 0.5 cc. of dilutions of each serum, 0.5 cc. of fresh guinea pig serum, diluted 1:10, was added which itself was not hemolytic under the conditions of the experiment. 1 drop (about 0.05 cc.) of 50 per cent washed sheep erythrocytes was added and the tubes were incubated at 37°C.

I. Injections of Mixtures of Extract and Serum.

Experiment 1 (Table I).—Materials injected into various batches of rabbits: (*a*) pig serum 1:10; (*b*) extract of horse kidney; (*c*) the same as (*a*), heated for ½ hour at 80°C.; (*d*) mixture of horse kidney extract and pig serum 1:10; (*e*) the same as (*d*), heated for ½ hour at 80°C. Tests after six intraperitoneal injections. Dilution of the rabbit sera 1:250. Period of incubation 1 hour.

TABLE I.

Material injected.	Pig serum (*a*).				Kidney extract (*b*).				Heated pig serum (*c*).			Mixture of kidney extract and pig serum (*d*).				Mixture of kidney extract and pig serum heated (*e*).			
Rabbit No.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Hemolysis.	0	0	0	F.Tr.	0	0	0	Tr.	0	0	0	C.	M.	Sl.	F.Tr.	Tr.	St.	Sl.	A.C.

In the tables C. indicates complete hemolysis; A.C., almost complete; V.St., very strong; St., strong; M., marked; Sl., slight; Tr., trace; F.Tr., faint trace; and 0, no hemolysis.

The experiment shows a marked difference in the response to the extract and to the mixture of extract and serum. There is no distinct difference between that to the heated and unheated mixtures. No hemolytic power of the sera was found after injections of pig serum in the dilution used.

Experiment 2.—Similar results were obtained after four intraperitoneal injections into three rabbits of alcoholic extract mixed with pig serum diluted 2.5 times. In this instance the mixture was faintly acidified with acetic acid and coagulated by heating for ½ hour to 75-80°C.

Experiment 3 (Table II).—Materials injected: (*a*) pig serum diluted 1:8; (*b*) mixture of kidney extract and 1:8 diluted pig serum. Tests after four intravenous injections. Dilution of the sera 1:250. Period of incubation 1 hour.

TABLE II.

Material injected.	Pig serum (*a*).				Mixture of kidney extract and pig serum (*b*).		
Rabbit No.....	23	24	25	26	27	28	29
Hemolysis.....	0	0	Tr.	0	0	C.	A.C.

As part of the same experiment four rabbits were injected subcutaneously with the mixture. The sera of these animals never manifested more than a slight hemolytic activity.

Experiment 4 (*Table III*).—Materials injected: (*a*) human serum diluted 1:8; (*b*) extract of horse kidney; (*c*) mixture of horse kidney and human serum diluted 1:8. Tests were made after five intravenous injections. Dilutions of the sera 1:500. Period of incubation 1 hour.

TABLE III.

Material injected	Human serum (*a*).											
Rabbit No.	34		35		36		37		38			
Hemolysis	0		Tr.		0		0		Tr.			
Material injected	Kidney extract (*b*).											
Rabbit No.	39	40	41	42	43	44	45	46	47	48	49	50
Hemolysis	0	Tr.	0	0	0	Sl.	0	Tr.	0	0	A.C.	0
Material injected	Mixture of kidney extract and human serum (*c*).											
Rabbit No.	51	52	53	54	55	56	57	58	59	60	61	62
Hemolysis	C.	C.	Sl.	C.	A.C.	C.	Tr.	C.	C.	C.	C.	C.

The dilutions up to which complete hemolysis took place are given in Table IV. According to these results the average titer is about eighteen times higher in the second series than in the first. Moreover, the experiment clearly shows some increase of the hemolytic power in a number of the animals injected with the extract alone. In only one of them is the titer remarkably high (No. 49), but in another experiment, not quoted here, two out of five animals showed a similar increase and one a still higher hemolytic activity. It should be mentioned that in these particular animals coccidiosis was found. It is uncertain whether or not this observation is of any significance.

With regard to the action of the extract alone, an opportunity was afforded in Experiment 4 of comparing in different stages of immunization the sera of rabbits injected with alcoholic extract. The tests were performed at the same time, one set of animals having received two, the other four injections. The dilution of the sera was 1:25, the incubation period 15 minutes. The results are given in Table V.

In addition to the hemolytic activity of the sera, the precipitating power was also tested in some of the experiments in which proteins were injected. It was found that frequently the most active hemolysins and precipitins were developed in the same animals, but this parallelism was not constant.

TABLE IV.

Material injected	Kidney extract (*b*).											
Rabbit No.	39	40	41	42	43	44	45	46	47	48	49	50
Complete hemolysis up to	1:<25	1:50	1:<25	1:<25	1:25	1:100	1:<25	1:100	1:25	1:<25	1:250	1:<25
Material injected	Mixture of kidney extract and human serum (*c*).											
Rabbit No.	51	52	53	54	55	56	57	58	59	60	61	62
Complete hemolysis up to	1:1,000	1:1,000	1:100	1:2,000	1:250	1:1,000	1:50	1:2,000	1:1,000	1:2,000	1:1,000	1:1,000

TABLE V.

No. of injections with kidney extract.	Two.						Four.					
Hemolysis	0	Tr.	0	0	Sl.	0	M.	A.C.	St.	0	St.	C.

II. Comparison between Injections of Mixtures and Separate Injections of the Components.

Although a definite increase of the hemolytic activity was not found after injection of either pig or human serum, the possibility had to be borne in mind that by injecting two weakly active substances; *i.e.* extract + serum, instead of a single one a summation of the effects could result. Therefore in the following experiments two series of rabbits were injected. The animals of the first series received 5 cc. of the mixture intravenously as in the preceding experiments. The animals of the second series received 5 cc. of the emulsion of kidney extract into one ear vein and 5 cc. of the diluted serum into the ear vein of the other side. Thus each animal of the two series received the same quantity of both substances, and if the effect had been due simply to an addition, both groups should have reacted in the same degree. This experiment is also related to another point. One might suppose that if a non-antigenic substance is injected into an animal during the period of immunization brought about by an antigen, the former substance would be included, as it were, in the process of immunization. The results obtained, however, do not support such a view.

Experiment 5 (*Table VI*).—Materials injected: pig serum diluted 1:8; and kidney extract. Tests after five intravenous injections. Rabbit sera diluted 1:500. Period of incubation 1 hour.

TABLE VI.

	Separate injections of extract and serum.								
Rabbit No.	63	64	65	66	67	68	69		
Hemolysis	0	0	0	V.St.	Sl.	C.	0		
Complete hemolysis up to						1:500			
	Mixture.								
Rabbit No.	70	71	72	73	74	75	76	77	78
Hemolysis	C.	V.St.	V.St.	A.C.	A.C.	C.	V.St.	C.	C.
Complete hemolysis up to	1:2,000					1:500		1:1,000	1:500

Experiment 6 (*Table VII*).—Materials injected: human serum diluted 1:8; and kidney extract. In addition to the injections of both components and the mix-

ture, a third series of five rabbits was injected intravenously with diluted human serum alone. Tests after five intravenous injections. Rabbit sera diluted 1:500. Incubation period 15 minutes and 1 hour.

In these two experiments the difference between the two sets of animals in which the same substances were injected in equal quantities but in different ways is very marked. The question as to whether injections of extract and serum made separately are more effective than the injections of extract alone has not yet been studied sufficiently to venture any statement.

TABLE VII.

	Human serum.						
Rabbit No.	79	80	81	82	83		
Hemolysis after 1 hr.	0	0	0	0	0		
	Separate injections of extract and serum.						
Rabbit No.	84	85	86	87	88	89	90
Hemolysis after 15 min.	0	0	0	0	0	M.	0
" " 1 hr.	0	Tr.	Tr.	0	F.Tr.	A.C.	M.
	Mixture.						
Rabbit No.	91	92	93	94	95	96	97
Hemolysis after 15 min.	C.	M.	V.St.	Sl.	A.C.	V.St.	0
" " 1 hr.	C.	A.C.	C.	V.St.	C.	C.	M.
Complete hemolysis up to	1:2,000		1:1,000		1:1,000	1:1,000	

DISCUSSION.

As the preceding experiments show, the antigenic power of heterogenetic antigen destroyed by the action of alcohol can be restored to a considerable extent by admixture of protein solutions such as serum. This effect can be demonstrated relatively easily, as the response of animals to heterogenetic antigen is more regular than with most other antigens, so that when the native antigen is used, antibodies can be obtained from almost every injected rabbit. However, the action of the artificial combination is not equal to that of the native antigen and more intensive treatment is required to produce the same result. Yet in this manner a satisfactory hemolytic titer is frequently obtained.

The related observations thus support the conception that the heterogenetic antigen is composed of two parts, as suggested above.

The supposition that a combination of the alcohol-soluble haptene with the added protein is, in our experiments, the acting substance seems to be the simplest interpretation of the results. The difference in the activity of the sera, when the mixture of haptene and protein is injected as such, or each component separately, demonstrates that some change results from mixing the two substances *in vitro*. The assumption that the effect of the injected mixture is due to some modification other than the formation of a compound seems hardly probable, since a mixture of the haptene with serum also occurs when haptene alone is injected intravenously. In the latter case, however, no marked effect follows. From our point of view this result is comprehensible because homologous proteins are much less adapted to antigenic action than foreign ones. They doubtless can be transformed into antigens[23] even by comparatively slight changes but are always inferior to materials of foreign origin. If doubt remain in the matter it could probably be tested by injections of the heterogenetic haptene mixed with rabbit serum.

With regard to the alcohol-soluble body itself, it is now almost generally admitted[24] that it has no antigenic power. This statement is approximately but not absolutely correct.[25] Observations on a considerable number of repeatedly injected rabbits leave no doubt as to the occurrence of a slight increase in hemolytic power in some of them, even if the few exceptional cases of high titer are ruled out. This finding can be attributed either to real immunization or to an enhanced output of normal antibodies, but the first mentioned explanation seems to be far more likely. There is reason then to believe that after injection of non-protein substances production of antibodies can take place,[26] if to but a slight degree. One can suppose that such an immun-

[23] Pick, R., and Obermayer, F. Landsteiner, K., and Jablons, B., *Z. Immunitätsforsch., Orig.*, 1913–14, xx, 618. Landsteiner, K., and Lampl, H., *Z. Immunitätsforsch., Orig.*, 1917, xxvi, 293.

[24] Sordelli and coworkers, Taniguchi, and von Gutfeld. *Cf.* Meyer, K., *Biochem. Z.*, 1921, cxxii, 225.

[25] *Cf.* Landsteiner, K., *Biochem. Z.*, 1921, cxix, 298, 304.

[26] K. Meyer, Kleinschmidt, Much, and others.

ization is due to the formation of compounds of the haptene with proteins of the rabbit, as in the experiments cited above, and a similar explanation may hold true for the other instances in which antigenic power is ascribed to lipoids, so called. As this view is only hypothetical, it does not enable one to make a sharp distinction between antigenic and non-antigenic substances. But in practise some discrimination is possible because of the wide differences in the activity of the two classes. The immunizing effects ascribed to non-protein substances, as, for example, fats or lipoids, are slight and irregular, and the only substances which can be used effectively for the preparation of antibodies are proteins, if bodies of unknown chemical nature such as toxins are excluded.[27] Consequently it may be assumed that a somewhat different mechanism is concerned in the two instances mentioned. In any case there would appear to be an intimate connection of proteins with the process of antibody production, which is probably not only dependent on the size of the molecules[28] but also on the chemical structure.

The fact that the addition of protein to a substance can transform it into an efficient antigen suggests similar investigation of other substances.[29]

CONCLUSIONS.

1. The alcohol-soluble extract of heterogenetic antigen, which possesses the specific chemical structure of the entire antigen, has a detectable but generally very slight power to increase the amount of heterogenetic antibodies, when injected into rabbits.

2. This substance can be transformed into an efficient antigen by mixing it with protein solutions such as diluted normal serum.

3. Such mixtures are considerably more active than the same substances injected separately. Therefore, the effect of the serum is probably due to the formation *in vitro* of a loose compound between the

[27] See Wells, H. G., Chemical pathology, Philadelphia and London, 2nd edition, 1914.

[28] Landsteiner, K., *Biochem. Z.*, 1919, xciii, 106.

[29] *Cf.* Doerr, R., *Schweiz. med. Woch.*, 1921, ii, 937; Schnabel, A., *Jahresk. ärztl. Fortbild.*, 1920, xi, 15.

alcohol-soluble substance and protein, the compound acting as a complete antigen.

4. It may be supposed that there exists a group of natural antigens which are built up of one specifically reacting part that is almost or entirely devoid of antigenic properties, and another part, a protein, responsible for the immunizing effect.