ON THE USE OF ADSORBENTS IN IMMUNIZATIONS WITH HAPTENS*

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Immunization with so called haptens, *i.e.* substances with definite serological activity but which with the usual laboratory methods exhibit rather weak or no immunizing capacity, was accomplished by Landsteiner and Simms (3), who obtained strong heterogenetic antisera following the injection of mixtures of Forssman's substance with human or pig serum. This method, which proved to be of general applicability, tended to emphasize the rôle of proteins in the production of immune sera to haptens, and under this assumption also showed that an immunization effect can be obtained without a firm chemical combination of hapten and protein.

Following this and the demonstration by Glenny (4), Ramon (5), and others that immunizations with toxins can be improved by adsorption to inert colloids, or the simultaneous injection of irritating substances, Gonzalez and Armangué (6) reported the interesting observation that extracts containing the heterogenetic substance can be made antigenic by adsorption to such materials as kaolin or charcoal. Doerr and Hallauer (7) had previously failed to immunize with mixtures of the heterogenetic extracts and charcoal. From this work of Gonzalez and Armangué it would appear that proteins can be effectually replaced by inert inorganic adsorbents in immunizations with haptens. The experiments of Gonzalez and Armangué have been confirmed by Landsteiner and Jacobs (1) with the Forssman antigen and recently by Plaut (8) with a hapten from brain.

In addition Zozaya (9) has reported considerable immunization effects with carbohydrates adsorbed to collodion particles and similar experiments with dextran, a carefully purified, nitrogen-free

* The experiments given in this paper have been presented preliminarily by Landsteiner and Jacobs (1, 2).

polysaccharide from *Leuconostoc mesenterioides*.

On the other hand, with a carbohydrate from rhinoscleroma bacilli adsorbed to collodion particles by Zozaya's method, Prášek (10) failed to obtain evidence of antibody formation.

The following experiments were undertaken with the object of obtaining additional information on the mode of action of inorganic adsorbents in hapten immunizations.

Technique

Horse Kidney Extract.—Two extracts were used; one made with 95 per cent alcohol at room temperature, and the other by heating for 1 hour on the steam bath. For immunizing with kaolin 5 cc. of the crude heterogenetic extract (extract from 250 gm. of horse kidney in 85 cc. of saline) diluted 1:4 with saline was mixed with 0.2 gm. of kaolin, and allowed to stand at least an hour at room temperature, before each injection.

For immunizing with collodion particles 50 cc. of the crude heterogenetic extract diluted 1:4 with saline was added to sedimented collodion particles in a quantity of approximately five billion to each cubic centimeter of the extract. After thorough mixing, standing for 1 hour at room temperature, and centrifuging, the centrifugate was made up to 200 cc. with saline and 0.25 per cent phenol added.

Forssman Substance A.—This was identical with substance A described by Landsteiner and Levene (11), giving a strong orcinol-copper test. For immunizing with kaolin, a solution of 150 mg. of the substance in 150 cc. of phenolized (0.25 per cent) saline was added to 6.0 gm. of kaolin. The mixture was allowed to stand an hour at room temperature with vigorous shaking at short intervals. For immunizing with pig serum 150 mg. of the Forssman substance were dissolved in 127.5 cc. of saline, 15 cc. of pig serum and 7.5 cc. of 5.0 per cent phenol added, and the solution well mixed.

Forssman Substance B.—Substance B was a preparation obtained by precipitation with copper sulfate in alkaline solution (11). For immunizing with kaolin 35 mg. of the preparation were dissolved by the addition of 17.5 cc. of 5.0 per cent phenol and gentle warming. This solution was diluted to 35.0 cc. with distilled water, 7.0 gm. of kaolin added and made up to a volume of 175 cc. with saline, followed by vigorous shaking at 5 minute intervals for a period of an hour. In immunizations with pig serum, 30 mg. of the Forssman preparation were dissolved in 7.5 cc. of 5.0 per cent phenol and added to a solution containing 18.7 cc. of pig serum and 123.8 cc. of saline.

Specific Substance from V. cholerae.—The organisms were grown, extracted, and treated with N/10 NaOH as described previously (12). The resulting substance gave a distinctly positive biuret reaction at a dilution of 1:50. For injections, 90.0 mg. of the substance were dissolved in 9.0 cc. saline, 6 gm. of blood charcoal

added, phenolized (final concentration 0.25 per cent), and made up to a volume of 150.0 cc. with saline. The mixture was allowed to stand 1 hour at room temperature, with vigorous shaking at 5 minute intervals. A precipitation test on the supernatant of this preparation, after centrifuging, showed that the adsorption was almost complete.

Heteroalbumose.—This preparation was obtained by peptic digestion of coagulated sheep serum, as described previously (13). It was soluble in saline. For injections with charcoal 100 cc. of a 1.0 per cent sheep heteroalbumose solution in saline were added to 20.0 gm. of blood charcoal and the suspension thoroughly mixed and allowed to stand $\frac{1}{2}$ hour at room temperature. 10 cc. of 5.0 per cent phenol were added and the volume made up to 200 cc. with saline. For injections with alum, to 100 cc. of 1.0 per cent sheep heteroalbumose solution in saline were added 10.0 cc. of 10.0 per cent alum potash followed by enough normal sodium carbonate to make the reaction slightly alkaline to litmus. The suspension was made up to 190 cc. with saline and 10.0 cc. of 5.0 per cent phenol added.

Adsorbents.—Acid-washed, biuret-free kaolin (Eimer and Amend) was used in these experiments, after washing with hot saline solution. Blood charcoal (Merck) was heated in a crucible and ground in a mortar before using. Collodion particles were prepared according to the method of Loeb (14).

Immunization.—For experiments on the production of hemolysins, rabbits were selected whose sera did not hemolyze sheep blood distinctly in a dilution of 1:25, under the conditions of the tests. Injections of 5.0 cc. each intravenously or intraperitoneally were administered at weekly intervals and the animals tested 7 days after the last injection. Injections of control solutions were always made with the same concentration and volume of solution, as was used with the adsorbents. Preparations with pig serum and collodion particles were injected intravenously; all other injections were intraperitoneal.

Precipitin Tests.—To 0.2 cc. of the antigen dilution in a small tube was added 2 drops of immune serum, and readings taken after 1 hour at room temperature, and overnight in the ice box. The strength of precipitation was recorded as follows: 0, f. tr. (faint trace), tr. (trace), ±, ±, +, +±, ++, etc.

Agglutinin Tests.—To 0.5 cc. of the given dilutions of inactivated serum was added 0.5 cc. of a saline suspension of killed cholera vibrios grown overnight on agar slants. The tests were read after 2 hours at 37°C. and overnight in the ice box, and the same symbols used in recording the readings, as in the precipitation tests. Sediment was described as sl. (slight), w. (weak), or dis. (distinct).

Hemolysis Tests.—To 0.5 cc. of saline dilutions of each serum was added 0.5 cc. of fresh guinea pig complement, diluted 1:10, which did not cause hemolysis, under the conditions of the test. 1 drop (about 0.05 cc.) of 50 per cent washed sheep erythrocytes was added and the tubes incubated for 1 hour at 37°C. Degrees of hemolysis were distinguished according to the following scheme: 0, tr. (trace), w. (weak), dis. (distinct), str. (strong), v. str. (very strong), a.c. (almost complete), c. (complete).

EXPERIMENTAL

The experiments of Gonzalez and Armangué were repeated without difficulty,[1] using emulsions of horse kidney extracts, as shown in Table I. A marked difference in the action of the preparations adsorbed to kaolin as compared to the controls was noticeable even after the second injection.

TABLE I

Crude Horse Kidney Extract. Tests Made after Five Injections

Material injected	Horse kidney extract				
Rabbit No.	26-57	26-60	26-62	26-67	26-73
Complete hemolysis to	<1:25	<1:25	<1:25	<1:25	<1:25
Material injected	Horse kidney extract adsorbed to kaolin				
Rabbit No.	26-59	26-64	26-66	26-71	26-73
Complete hemolysis to	1:25	1:400	1:1600	1:25	1:200

TABLE II

Crude Horse Kidney Extract. Tests Made after Five Injections

Material injected	Unheated horse kidney extract adsorbed to collodion particles				
Rabbit No.	18-94	18-95	18-96	18-97	18-98
Complete hemolysis to	<1:25	<1:25	<1:25	<1:25	1:50
Material injected	Heated horse kidney extract adsorbed to collodion particles				
Rabbit No.	18-99	19-00	19-01	19-02	19-03
Complete hemolysis to	1:50	1:50	1:200	1:25	1:50

Although this was not the case in experiments with kaolin, with apparently less suitable adsorbents, such as collodion particles, crude heterogenetic extracts seemed to give better results when prepared by heating on the steam bath then when the extraction was performed at

[1] Weil and Berendes (15) who, like Misawa (16), reported negative experiments, note that their failure was associated with a heavy loss of animals due to the injection of kaolin. We, also, observed fatalities with injections of 0.5 gm., but had no difficulty with injections of 0.2 gm.

room temperature (Table II). In both cases, the fact that adsorption took place was shown by lysin inhibition using the adsorbed collodion particles and supernatants. With turpentine, injected intraperitoneally in a quantity of 0.1 cc. immediately following the injection of the Forssman extract, there was a similar but still less marked result.

Unheated extracts injected alone gave rise to practically no

TABLE III

Forssman Preparation A. Tests Made after Three Injections

Material injected	Forssman preparation A adsorbed to kaolin (orcin substance)				
Rabbit No	30-86	31-51	31-52	31-53	31-54
Complete hemolysis to	<1:25	<1:25	<1:25	<1:25	<1:25
Material injected	Forssman preparation A mixed with pig serum				
Rabbit No	30-84	30-85	31-48	31-49	31-50
Complete hemolysis to	1:800	1:50	1:100	1:100	1:400

TABLE IV

Forssman Preparation B. Tests Made after Five Injections

Material injected	Forssman preparation B adsorbed to kaolin				
Rabbit No	19-04	19-05	19-06	19-07	19-08
Complete hemolysis up to	<1:25	<1:25	<1:25	<1:25	<1:25
Material injected	Forssman preparation B mixed with pig serum				
Rabbit No	29-10	29-11	29-12	29-13	29-14
Complete hemolysis up to	1:800	1:200	1:800	1:800	1:320

hemolysin formation, while heated extracts alone yielded sera which were apt to give complete hemolysis at dilutions of 1:25 after four or five injections, but not in higher dilutions. An experiment in which heterogenetic antigen was injected intravenously and the usual amount of kaolin intraperitoneally, failed to show activation.

With the Forssman preparations designated as A and B which contained smaller quantities of extraneous material than the crude ex-

tract, no hemolysin formation was obtained, using kaolin. As is evident from Tables III and IV, however, these same preparations, when mixed with pig serum, were active in producing hemolytic sera.

Adsorption to kaolin was tested by lysin inhibition with the adsorbed kaolin preparations and the supernatants. Both Forssman substances

TABLE V

Crude Cholera Carbohydrate. Tests Made after Five Injections

Injected with	Rabbit No.	Agglutination, serum diluted						Precipitation	Read after
		1:50	1:100	1:200	1:400	1:800	1:1600	Dilution of antigen 1:5000	
									hrs.
Cholera preparation alone	26-46	0						0	2
		sl. sed.						0	24
	26-47	0						0	2
		dis. sed.						0	24
	26-48	0						0	2
		dis. sed.						0	24
Cholera preparation adsorbed to charcoal	26-49	+±	+	±	±	tr.	f. tr.	+±	2
		+±	+	tr.	dis. sed.	w. sed.	sl. sed.	+±	24
	26-50	+	tr.	0	0			tr.	2
		tr.	dis. sed.	w. sed.	0			±	24
	26-51	tr.	0	0	0			f. tr.	2
		dis. sed.	sl. sed.	0	0			f. tr.	24
	26-52	±	tr.	0	0			tr.	2
		±	dis. sed.	w. sed.	sl. sed.			tr.	24
	25-43	tr.	0	0	0			f. tr.	2
		w. sed.	sl. sed.	0	0			f. tr.	24

were largely adsorbed.[2] Preparation A was administered in injection doses of 5 mg. each, and preparation B in amounts of 1 mg. per

[2] Adsorption of Forssman antigen to kaolin has been studied by Fischer (17), Weil and Berendes (15), Rudy (18), and others.

injection. With the latter substance a second experiment confirmed the first. With a third Forssman preparation (19), adsorbed to kaolin, attempts at immunization failed in a similar manner. These experiments furnish evidence that the injection of kaolin alone does not lead to the production of hemolytic sera, which is worth mentioning because Misawa (16) reported the production of hemolytic sera following injections of charcoal. He states, however, that his results

TABLE VI

Sheep Heteroalbumose. Precipitin Tests Made after Eight Injections

Substance injected	Rabbit No.	Heteroalbumose 1:2000	
		2 hrs.	24 hrs.
Heteroalbumose	29-30	0	0
	29-31	0	0
	29-32	0	0
	29-33	0	0
	29-34	0	0
Heteroalbumose adsorbed to alum	29-35	tr.	±
	29-36	0	tr.
	29-37	tr.	+
	29-38	tr.	±
	29-39	0	0
Heteroalbumose adsorbed to charcoal	29-40	f. tr.	±
	29-41	0	tr.
	29-42	±	+
	29-43	tr.	+
	29-44	+	+±

were uncertain, due to the possible presence of a *lepisepticus* infection in the rabbits.

With a cholera carbohydrate preparation which still gave a positive biuret reaction, adsorbed to charcoal, there was a marked increase in antigenicity over that shown by the carbohydrate preparation alone, as is evident in Table V. The controls in the agglutination test were not entirely negative, since they showed on standing overnight in the ice chest slight to distinct sedimentation after the third and subsequent injections.

With a carbohydrate preparation from the same organism which had been treated similarly with alkali but from which the protein was removed almost completely by heating with acetic acid so that the biuret reaction was but very faintly positive, one experiment with charcoal yielded no evidence of antibody formation whatever, and a second, one slight and one faint reaction. Five rabbits were used in each of these experiments.

Two preparations of dextran[3] adsorbed to collodion particles and injected by Zozaya's technique, failed to produce demonstrable antibodies. Two other entirely or practically protein-free carbohydrates, one from Pneumococcus Type I, and the other from a pseudoanthrax bacillus, adsorbed to charcoal and injected into rabbits, gave negative results. Thus attempts to demonstrate an increase in precipitin or agglutinin formation following injections of carbohydrates which had been freed, to a large extent, from protein, were not successful.

The heteroalbumose preparation used was known (20) to be a weak antigen with which sera of workable titer could be obtained only with difficulty. In the experiment shown in Table VI, comparable injections of heteroalbumose, alone and adsorbed either to charcoal or to alum, showed markedly increased antibody formation with the use of these adsorbents, which has been of practical advantage in the preparation of antisera to this material (21). In this experiment, the sera of the control rabbits, injected with heteroalbumose alone, happened to be entirely negative.

DISCUSSION

In confirmation of the experiments of Gonzalez and Armangué we obtained very active heterogenetic immune sera with the aid of adsorbents. The enhancement of immunizing activity which takes place after adsorption to kaolin occurs whether the horse kidney extracts used are prepared by heating or by standing at room temperature. In the inconstant activations which followed adsorption to collodion particles or the simultaneous injection of turpentine, only the heated extracts showed a slight enhancement. These latter extracts were more highly colored than those obtained by standing at room tempera-

[3] For supplying this substance we are indebted to the kindness of Dr. Harold Hibbert of Montreal.

ture, and caused slight hemolysin formation when injected alone, suggesting that they may contain more impurities than the unheated ones.

Experiments carried out with certain fractions of the original extracts gave results which seem to offer information on the activation by adsorbents. Forssman preparations which had undergone treatments designed to remove inactive material, although highly active in tests with immune sera, were no longer able to produce hemolysins on adsorption to kaolin. On the other hand, hemolysins appeared when these substances were injected along with pig serum. With this hapten preparation, therefore, we did not succeed in substituting kaolin for foreign serum, and the most obvious explanation would appear to be that some impurity is active in the enhancement of antigenicity by adsorbents. In regard to this it is of interest to note that Plaut (8), who was able to produce immune sera by the injection of brain hapten adsorbed to aluminum hydroxide, did not succeed in obtaining such an effect with a chemically pure substance, namely cholesterin, with which, however, antibodies can be produced, according to several authors, by admixture with antigenic protein.

The experiments with carbohydrates point in a similar direction. A preparation obtained from *V. cholerae* by extraction with 75 per cent alcohol and treatment with alkali, gave a strong biuret reaction and moderately strong agglutinin and precipitin formation in rabbits after adsorption to charcoal. Further treatment with protein precipitants resulted in a substance giving a negative or exceedingly faint biuret, and of very slight immunizing activity after adsorption. Carefully purified carbohydrates from *B. pseudoanthracis* and Pneumococcus Type I adsorbed to charcoal, and two different preparations of dextran[4] adsorbed to collodion particles and to charcoal, gave practically negative results. It would seem that with the methods tried the carbohydrates used have little or no capacity for engendering the production of precipitating antibodies, although this does not rule out the presence of such slight activity as would be necessary to give positive results in more delicate tests; *e.g.*, the production of active immunity to infections, or skin reactions in human beings following

[4] *Cf.* Zozaya, J., *J. Exp. Med.*, 1932, **55**, 346.

intracutaneous injections. In this connection mention should be made of the experiments of Tillett and Francis (22), Schiemann and his collaborators (23), Enders (24), Sabin (25), Wadsworth and Brown (26), Ward (27), Felton (28), and the work by Avery and Goebel (29) and Francis (30) on the immunizing properties of the polysaccharides of Pneumococcus Type I.

That an increase in the output of antibodies such as Gonzalez and Armangué and Zozaya[5] have reported may be obtained by the use of adsorbents is supported by the work of Glenny on alum-toxin mixtures. Further evidence is afforded by the heteroalbumose experiment described, in which the immunizing effect was enhanced considerably since a preparation of heteroalbumose with which it was rather difficult to produce antisera gave more active sera when adsorbed to alum or charcoal.

For the immunization effects following adsorption to inorganic colloids several explanations may be considered. The adsorbent may serve as a vehicle by means of which the substances are taken up by cells or it may slow down their elimination or bring about a stronger inflammatory reaction. In support of the latter is the fact that the peritoneal cavity is markedly inflamed following the injection of the usual amount of kaolin (0.2 gm.). Since Forssman extracts are not always activated by adsorption to collodion particles, it would appear that to administer the material in the form of particles does not necessarily suffice to increase antigenicity. In the case of immunization effects obtained with foreign sera (and not with homologous serum), the antigenic property of the serum would still seem to play the decisive rôle.

One may infer that the effects observed following adsorptions such as those of Gonzalez and Armangué, and of Zozaya in so far as they can be reproduced, are also due to an increase of preexistent antigenic activity which might be attributed either to an antigenic function of the haptens themselves (31) or else to the concurrent action of other substances either mixed or combined with the haptens.

[5] In a later paper (*J. Exp. Med.*, 1933, **57**, 38) Zozaya tentatively explained marked differences observed in the antigenicity of various polysaccharides on the assumption that the carbohydrates had lost their immunizing property in the process of purification while retaining their serological activity.

SUMMARY

Experiments are described which confirm the observation of Gonzalez and Armangué that heterogenetic extracts can be made antigenic by adsorption to inorganic materials. With fractions of the original extracts from which a part of inactive material had been removed no such enhancement was observed, whereas with foreign protein an activation was still possible. Carbohydrate preparations behaved similarly in that purification, perhaps loss of protein, was accompanied by a distinct decrease in antigenicity after adsorption. The activity of a but slightly antigenic heteroalbumose preparation was markedly increased after adsorption to charcoal and alum. The most reasonable explanation for the effects observed by Gonzalez and Armangué, and Zozaya, seems to be that a preexisting antigenic capacity has been enhanced by the use of adsorbents. The experiments reported here support the view that these effects are influenced significantly by the presence of substances other than those of a specific nature.

REFERENCES

1. Landsteiner, K., and Jacobs, J., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29,** 570.
2. Landsteiner, K., and Jacobs, J., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30,** 1055.
3. Landsteiner, K., and Simms, S., *J. Exp. Med.*, 1923, **38,** 127.
4. Glenny, A. T., Pope, C. G., Waddington, H., and Wallace, U., *J. Path. and Bact.*, 1925, **28,** 463; 1926, **29,** 31; 1928, **31,** 403. Glenny, A. T., and Barr, M., *J. Path. and Bact.*, 1931, **34,** 118, 131. Glenny, A. T., Buttle, G. A. H., and Stevens, M. F., *J. Path. and Bact.*, 1931, **34,** 267.
5. Ramon, G., *Compt. rend. Soc. biol.*, 1925, **93,** 506.
6. Gonzalez, P., and Armangué, M., *Ier Cong. Int. Microbiol.*, Paris, 1930, **2,** 42; *Compt. rend. Soc. biol.*, 1931, **106,** 1006; 1932, **110,** 217, 220. Gonzalez, P., Armangué, M., and Romero, S., *Compt. rend. Soc. biol.*, 1932, **110,** 223. Gonzalez, P., Armangué, M., and Morato, T., *Compt. rend. Soc. biol.*, 1932, **110,** 216, 228. Gonzalez, P., and Armangué, M., *Am. J. Hyg.*, 1933, **17,** 277.
7. Doerr, R., and Hallauer, C., *Z. Immunitätsforsch.*, 1926, **47,** 310.
8. Plaut, F., and Rudy, H., *Z. Immunitätsforsch.*, 1933, **81,** 87.
9. Zozaya, J., *Science*, 1931, **74,** 270; *J. Exp. Med.*, 1932, **55,** 325, 353; Zozaya, J., and Clark, J., *J. Exp. Med.*, 1933, **57,** 21.
10. Prášek, E., and Prica, M., *Centr. Bakt., 1. Abt., Orig.*, 1933, **128,** 381.
11. Landsteiner, K., and Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1927, **24,** 693.
12. Landsteiner, K., and Levine, P., *J. Exp. Med.*, 1927, **46,** 213.

13. Landsteiner, K., and van der Scheer, J., *Z. Hyg. u. Infektionskrankh.*, 1931, **113,** 2.
14. Loeb, J., *J. Gen. Physiol.*, 1922–23, **5,** 111.
15. Weil, A. J., and Berendes, J., *Z. Immunitätsforsch.*, 1931–32, **73,** 341.
16. Misawa, T., *Z. Immunitätsforsch.*, 1933, **79,** 80.
17. Fischer, T., *Z. Immunitätsforsch.*, 1933, **79,** 39.
18. Rudy, H., *Klin. Woch.*, 1932, **11,** 1432.
19. Landsteiner, K., and Levene, P. A., *J. Immunol.*, 1925, **10,** 731.
20. Landsteiner, K., and van der Scheer, J., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28,** 983.
21. Landsteiner, K., and Chase, M. W., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30,** 1413.
22. Tillett, W. S., and Francis, T., Jr., *J. Exp. Med.*, 1929, **50,** 687.
23. Schiemann, O., and Casper, W., *Z. Hyg. u. Infektionskrankh.*, 1927, **108,** 220. Schiemann, O., Loewenthal, H., and Hackenthal, H., *Z. Hyg. u. Infektionskrankh.*, 1931, **112,** 315.
24. Enders, J. F., *J. Exp. Med.*, 1930, **52,** 235; 1932, **55,** 191. Pappenheimer, A. M., and Enders, J. F., *Proc. Soc. Exp. Biol. and Med.*, 1933, **31,** 37.
25. Sabin, A. B., *J. Exp. Med.*, 1931, **52,** 93.
26. Wadsworth, A., and Brown, R., *J. Immunol.*, 1931, **21,** 245; 1933, **24,** 349.
27. Ward, H. K., *J. Exp. Med.*, 1932, **55,** 519.
28. Felton, L. D., *J. Immunol.*, 1932, **23,** 405; *J. Infect. Dis.*, 1928, **43,** 531.
29. Avery, O. T., and Goebel, W. F., *J. Exp. Med.*, 1933, **58,** 731.
30. Francis, T., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31,** 493.
31. Landsteiner, K., Die Spezifizität der serologischen Reaktionen, Berlin, Julius Springer, 1933, 54.