

EXPERIMENTS ON ANAPHYLAXIS TO AZOPROTEINS THIRD PAPER

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(Received for publication, May 23, 1930)

In preceding papers (1, 2) it has been shown to be possible to induce anaphylaxis in guinea pigs by injecting azoproteins, namely, horse serum coupled with diazotized *p*-arsanilic acid. Animals treated in this way become sensitive to azoproteins containing the same azocomponent but another sort of protein; thus the reactions obviously depend upon the arsanilic acid group.

These results have been confirmed by Meyer and Alexander (3) who used also the method of passive sensitization, and by Klopstock and Selter (4). Some of the observations of these workers will be discussed later. Another investigation on the subject has been made recently by Tillett, Avery and Goebel (5). These authors employed as antigens azoproteins prepared by combining globulin or albumin with diazotized glucosides.

The present study was undertaken with the purpose of investigating the specificity of the anaphylactic reactions and of gaining further information on the inhibition phenomenon produced by simple chemical substances (2). In order to test the specificity of anaphylaxis to azoproteins, antigens were selected which differed only in the steric configuration of the specifically reacting groups. The fact that antigens containing sterically isomeric groups are serologically different has already been shown by means of the precipitin reaction by Landsteiner and van der Scheer (6,7) for *l*- and *d*-phenyl (*p*-aminobenzoylamino) acetic acids and *l*-, *d*-, and *m*-tartaric acids. Similar results were obtained by Avery and Goebel (8, 9) who used as antigens glucosides containing glucose or galactose.

EXPERIMENTAL

Specificity of the Anaphylactic Reaction.—For the following experiments antigens were used containing the radicals of the *d*- and *l*-tartaric acids. These were prepared by coupling horse serum with *d*- and *l*-*p*-aminotartranilic acid in the manner previously described (7).

For the sensitization guinea pigs were injected intraperitoneally three times at weekly intervals with a suspension of 1 cc. of the antigens containing about 5 per cent protein. The animals weighed 210 to 250 gm.; the injections were well tolerated.

The solutions used for the reinjection were prepared in the same manner as the sensitizing antigens except that chicken serum was employed instead of horse serum and that after precipitation with acid, the azoproteins, without treatment with alcohol, were brought in solution with the aid of sodium carbonate; the solutions were made isotonic and adjusted to litmus neutrality. The stock solutions of the antigen were brought to a protein content of 3.5 per cent. The test injections were made intravenously 3 weeks after the last administration of the sensitizing dose, with 1 cc. of various dilutions or a larger volume of the concentrated antigen. At the time of the test the weight of the animals was about 400 gm. The results of an experiment in which the specificity of the reaction was tested are given in Table I.

From Table I it is seen that, with one possible exception, the sensitization succeeded regularly; one animal showed only slight symptoms. The quantity sufficient for inducing shock was as low as 0.35 to 0.7 mg. The symptoms were in all cases typical of anaphylactic shock, and in the animals which died, the lungs were distended and the heart was beating. The reactions were strikingly specific since an injection of about 50 to 100 minimal lethal doses was innocuous for the animals sensitized to the heterologous antigen, apart from a drop in temperature which generally did not exceed 1°. In this respect the results are in full agreement with those reported by Tillett, Avery, and Goebel (5).

A further proof of the specificity of the reactions was furnished by reinjecting, on the following day, some of the animals which had received a dose of the heterologous antigen without showing symptoms of anaphylactic shock. Such animals, with one exception, reacted to

a subsequent injection of the same quantity of the homologous antigen, although there was evidence of some protection (Table II).

TABLE I¹
Animals Sensitized with d-Antigen

Tested with <i>d</i> -antigen				Tested with <i>l</i> -antigen			
Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.				°C.	
1	70		† 4 min.	14	70	-0.7	Negative
2	35		† 3 "	15	70	-0.4	"
3	35	-4.3	Severe	16	35	-1.5	Slight
4	35		† 3 min.	17	35	-0.7	Negative
5	35		† 3 "	18	17.5	-0.4	"
6	35		† 3 "	19	8.8	-0.9	"
7	35		† 5 "	20	0.7	+0.9	"
8	17.5	-3.3	Severe				
9	17.5		† 4 min.				
10	8.8		† 3 "				
11	1.5		† 4 "				
12	0.7	-2.3	Very severe				
13	0.35	-0.6	Moderate				

Animals Sensitized with l-Antigen

Tested with <i>d</i> -antigen				Tested with <i>l</i> -antigen			
Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.				°C.	
21	70	+1.0	Negative	26	70		† 4 min.
22	35	-1.1	"	27	35	-2.8	Slight
23	8.8	-1.9	"	28	35		† 3 min.
24	3.0	-0.5	"	29	8.8		† 3 "
25	0.7	-0.6	"	30	3.0		† 5 "
				31	0.7		† 5 "
				32	0.35	-2.4	Severe
				33	0.18	-1.3	Slight

¹ The designations correspond to those in the previous paper (7).

† Death of animal.

Guinea pigs which were sensitized with only one injection of 1 cc. of the antigen showed the same degree of sensitivity as those in the

TABLE II
Reinjection Experiments
Animals Sensitized with d-Antigen

Guinea pig No.	Quantity of <i>l</i> -antigen injected in mg.	Result, symptoms	Quantity of <i>d</i> -antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
17	35	Negative	35	°C. -1.8	Negative
18	17.5	"	17.5		† 5 min.
19	8.8	"	8.8	-2.5	Severe
20	0.7	"	0.7		† 8 min.

Animals Sensitized with l-Antigen

Guinea pig No.	Quantity of <i>d</i> -antigen injected in mg.	Result, symptoms	Quantity of <i>l</i> -antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
21	70	Negative	70	°C.	† 8 min.
22	35	"	35		† over night
24	3	"	3	-2.2	Severe
25	0.7	"	0.7		† 5 min.

TABLE III

Guinea pigs weighing 200 to 220 gm. were given one intraperitoneal injection of *d*-antigen: reinjection with shocking antigen at the end of 23 days when the animal weighed about 300 gm.

Guinea pig No.	Quantity of <i>d</i> -antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
34	3	°C.	† 3 min.
35	0.7		† 4 "
36	0.7		† 5 "
37	0.35	-0.7	Moderate to severe

experiment reported in Table I since they also succumbed to a shocking dose of 0.7 mg. An example is given in Table III.

The above experiments furnished hardly any evidence of the inhibition of the anaphylactic reaction by the administration of large doses of the shocking antigen, which was observed by Klopstock and Selter (4). However there was some indication of the zone phenomenon in experiments (Table IV) in which animals were sensitized with an antigen made by coupling beef serum with diazotized arsanilic acid as was done by Klopstock and Selter.

It is seen from Table IV that only one of three animals was killed

TABLE IV

Eleven guinea pigs were sensitized by one subcutaneous injection of atoxyl beef antigen (1 cc. = 16.6 mg.) which was purified by means of acid and alcohol; the reinjection with atoxyl chicken antigen prepared in the same manner as the beef antigen was made 16 days after the sensitization. The reinjection was made intravenously in a volume of 1 cc.

Guinea pig No.	Quantity of chicken antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.	
38	6	-6.2	Very sick
39	6	-1.1	Negative
40	6		† 5 min.
41	1.5	-1.8	Slight to moderate
42	1.5		† 4 min.
43	1.5		† 7 "
44	1.5		† 7 "
45	1.5		† 6 "
46	0.5	-1.3	Slight to moderate
47	0.5		† 5 min.
48	0.5		† 6 "

by the largest dose employed while four out of five succumbed to a dose four times smaller.

Sensitization with Antigens Prepared According to the Method of Klopstock and Selter.—Whilst in the first experiments on anaphylaxis to azoproteins, the antigens used for sensitization were isolated after coupling in alkaline solution by precipitation with acid, Klopstock and Selter sensitized guinea pigs by injecting guinea pig serum to which they added neutralized diazosolutions. The reinjections were made with azoproteins prepared from guinea pig serum according to the older

method. In the experiment to be described (Table V) the procedure of Klopstock and Selter was followed.

The results as judged from the reinjection with the chicken serum preparation confirmed in a general way those of Klopstock and Selter except that the sensitization did not succeed regularly. They differed in that most of the animals tested did not react to the guinea pig serum

TABLE V

Eighteen guinea pigs were sensitized by a subcutaneous injection of 1 cc. of a solution made by adding two volumes of 1 per cent neutralized solution of diazotized *p*-arsanilic acid to one volume of fresh guinea pig serum (4). The solution stood overnight in the ice box before injections were made. The reinjections were made after an interval of 33 days.

Reinjection with chicken <i>p</i> -arsanilic acid antigen (1 cc. = 18.6 mg. protein)				Reinjection with guinea pig <i>p</i> -arsanilic acid antigen (1 cc. = 23.8 mg. protein)			
Guinea pig No.	Quantity of antigen in mg.	Subsequent change in body temperature °C.	Result, symptoms	Guinea pig No.	Quantity of antigen in mg.	Subsequent change in body temperature °C.	Result, symptoms
49	9.0		† 4 min.	59	12	-1.2	Negative
50	9.0	-1.3	Slight	60	12	-0.4	"
51	5.0		† 3 min.	61	5	-1.5	Slight to moderate
52	5.0		† 20 "	62	5		† 4 min.
53	5.0	-0.3	Negative	63	5	-0.2	Negative
54	5.0		† 20 min.				
55	5.0	-1.0	Negative				
56	1.5	-0.3	"	64	1.5	-0.6	"
57	1.5		† 6 min.	65	1.5	-1.8	"
58	1.5	-0.8	Negative	66	1.5	-0.6	"

preparation. No attempt was made to inquire into the cause of this discrepancy.

From their observations Klopstock and Selter conclude that for the sensitization and the production of antibodies, as well as for the reactions *in vitro*, it is not necessary to have a chemical combination of the azocomponents with protein but that it suffices to use simple "mixtures" of diazocompounds and protein. They stress the view that the diazocompounds would, by themselves, act as antigens and the proteins only enhance the antigenic activity which is inherent in

the simple substances (4, 10, 11). However, as has been shown by Heidelberger and Kendall (12) and one of the present writers (13), there undoubtedly takes place a combination of the diazocompounds with proteins also in neutral solution under the conditions of the method of Klopstock and Selter. Consequently their procedure does not involve a new principle but must be looked upon as a modification of the older method of preparing azoproteins. It is true that in a footnote to their last publication¹ the authors make a statement which may be interpreted as an admission that they dealt not with mixtures but with chemical combinations. As for those instances in which Klopstock and Selter succeeded in sensitizing with diazocompounds alone, it can be assumed that these substances combined with the proteins of the animal injected, so that in this case also the immunization is probably attributable to an azoprotein. Indeed it has been shown that animals can be immunized by azoproteins the protein part of which is derived from their own species (14).

The phenomenon observed by Klopstock and Selter, that guinea pigs sensitized with diazolutions alone exhibit skin reactions on intradermal injections of the same diazocompound, may be due to a special mechanism if further study should show that the skin reactions can be induced in this way only and not by sensitization with azoproteins. One may suppose either that the chemical combinations formed in the body on the injection of diazolutions are different from those prepared *in vitro* or that the sensitization is brought about by the direct action on tissues (skin) by the diazocompound as such. Even in the latter case, because of the ease with which diazolutions combine with proteins it would not be justifiable to draw conclusions, from the experiments discussed, upon the possibility of sensitization with simple chemical substances in general, particularly those which do not readily form compounds with proteins.

Inhibition of Anaphylactic Shock by Simple Substances.—In the experiments of Landsteiner (2) a peculiar phenomenon was noticed, *i.e.* shock could be prevented by injecting sensitized animals with simple azocompounds containing the same specific group as the sensitizing antigen. The substances used were compounds prepared

¹ See (4), page 465.

by coupling diazotized arsanilic acid with tyrosine or *p*-oxybenzoic acid. Analogous results were described by Klopstock and Selter (4) with the sodium salts of *p*-arsanilic acid (atoxyl) and *m*-aminobenzenesulfonic acid. K. Meyer did not succeed in obtaining antianaphylaxis by injecting atoxyl into animals sensitized to azoproteins prepared from *p*-arsanilic acid.

Similar experiments were carried out by Tillett, Avery, and Goebel (5) with guinea pigs sensitized to azoproteins containing glucosides. When these animals were injected with uncombined glucoside immediately prior to the administration of the antigen, shock could be prevented. If, however, the shocking injection was given 2 hours later protection was no longer demonstrable. Consequently the authors raise the question as to the mechanism of this inhibitory effect which, indeed, can hardly be looked upon as a desensitization on account of its transitory nature.

Our present experiments were carried out with a series of animals sensitized with antigens prepared from *d*- and *l*-*p*-aminotartranilic acid and another series sensitized to azoproteins prepared from *p*-arsanilic acid.

Guinea pigs weighing 200 to 250 gm. were sensitized as in the previous experiment (see Table I) and were tested 3 weeks after the last injection. At various intervals before the administration of the shocking homologous antigen the animals were injected with solutions of an azocompound made by coupling resorcinol with diazotized *d*- and *l*-*p*-aminotartranilic acids. The products are designated as *d*-T.R. and *l*-T.R., respectively.

These substances were prepared as follows: 480 mg. *d*- or *l*-*p*-aminotartranilic acid were diazotized in the usual way (7, page 410) and coupled with 110 mg. resorcinol. The dye formed was precipitated with the aid of dilute hydrochloric acid, the precipitate washed in acidulated water and dried.

A 1 per cent solution of the dye was made in a 0.9 per cent salt solution by adding dilute sodium hydroxide, and the solution was adjusted to neutrality or faint alkalinity. 1 cc. of various dilutions was injected intravenously. The shocking antigen was injected in a quantity of 7 mg. (1 cc. of a 0.7 per cent dilution) which corresponds to 5 to 10 minimal lethal doses (see Table I). At the time of the tests the weight of the guinea pigs was about 400 gm.

The experiments (Table VI) demonstrate that with one exception in which the animal showed severe anaphylactic symptoms, the guinea pigs previously injected with the heterologous azodye died in typical

TABLE VI
Inhibition by the Injection of Azodyes
Animals Sensitized with d-Antigen

Guinea pig No.	d-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of d-antigen	Subsequent change in body temperature °C.	Guinea pig No.	l-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of d-antigen	Subsequent change in body temperature °C.
67	10	1	Weakness	-4.5	77	10	1	† 4 min.	
68	10	18	Cough, slight weakness	-1.1	78	10	18	† 4 "	
					79	5	1	† 4 "	
					80	5	2½	† 4 "	
69	10	18	Dyspnea spasms	-1.2	81	5	2½	† 5 "	
					82	5	3½	† 4 "	
70	5	1½	Weakness	-0.2	83	5	24	† 5 "	
71	5	2	Negative	+0.7	84	2.5	1	† 5 "	
72	5	2½	Slight	-0.7	85	2.5	2½	Few convulsions, very sick	-3.6
73	5	2½	Negative	-0.3					
74	5	24	Cough	-4.0					
75			Weakness						
75	2.5	1	Somewhat sick, weakness	-1.3					
76	2.5	2	Negative	+0.6					

Animals Sensitized with l-Antigen

Guinea pig No.	d-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of l-antigen	Guinea pig No.	l-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of l-antigen	Subsequent change in temperature °C.
86	10	16	† 4 min.	96	10	16	Slight, weak	-2.8
87	5	2½	† 4 "	97	10	16	Negative	-1.0
88	5	3	† 4 "	98	5	2½	Negative	-0.2
89	5	3	† 4 "	99	5	3	† 5 min.	
90	5	3	† 5 "	100	5	3	Somewhat weak	-1.0
91	5	3	† 2 "					
92	5	3	† 5 "	101	5	3	Weakness	-1.9
93	5	3	† 4 "	102	5	3	Slight, weak	-0.5
94	5	3½	† 4 "	103	5	3	Weak	-1.8
95	5	4	† 6 "	104	5	3	Negative	-0.4
				105	5	3½	Slight, weak	-1.6
				106	5	3½	Slight, weak	-1.6

acute shock, whilst all animals but one, injected with quantities of 2.5 to 10 mg. of the homologous dye, survived. The surviving animals exhibited but rarely typical anaphylactic symptoms as spasms or cough, although many appeared sick. With higher doses of the dye the protection was still evident even when the injection of the antigen was made the following day.

Another batch of animals was sensitized passively by injecting a potent precipitating immune serum produced in a rabbit by immunization with azoprotein made from horse serum and diazotized *p*-arsanilic acid.² On injecting intraperitoneally guinea pigs weighing about 300 gm. with 0.3 cc. of this immune serum, the animals proved to be sensitive to an azoprotein prepared from diazotized *p*-arsanilic acid and chicken serum. The minimal lethal dose was regularly found to be 0.5 mg. In the tests presented in Table VII the animals were passively sensitized with 0.3 cc. of the immune serum. The substance tested for inhibition was a product of coupling diazotized *p*-arsanilic acid and tyrosine (2). This was injected intravenously in a volume of 0.5 cc. at stated intervals prior to the administration of the antigen or in a mixture with antigen (indicated as "0" in Table VII). The antigen was employed in a quantity corresponding to two minimal lethal doses.

From Table VII it is seen that the animals were protected from lethal shock by quantities of 2.5 to 1.25 mg. of the dye regardless of the time interval between the two injections. These animals showed either no symptoms or became somewhat weak. In two animals only were slight convulsions or coughing observed. With 0.6 and 0.3 mg. of the dye the results were irregular but again the outcome appeared not to depend on the time elapsed after the injection. A still smaller dose (0.15 mg.) failed to prevent shock also when the dye was injected simultaneously with the antigen.

A few experiments with solutions of sodium *p*-arsanilate seemed to indicate that also this substance has an inhibitory effect upon the anaphylactic reaction but considerably larger quantities were used than of the azosubstance.

The protection described can be explained in two ways, either by assuming that this effect is similar to the inhibition of the precipitin

² For the method see (15).

reaction *in vitro* by simple substances containing the specific group (16), or that the mechanism is analogous to the well known desensitiza-

TABLE VII

Inhibition by the Injection of Azodye

Animals passively sensitized with 0.3 cc. immune serum; the next day injection of the compound of *p*-arsanilic acid and tyrosin, followed by the administration of 1 mg. of the shocking antigen.

Guinea pig No.	Atozyl-tyrosine in mg.	Interval between injection of dye and antigen in hours	Shocking antigen	Result and symptoms after injection of shocking antigen	Subsequent change in body temperature
					°C.
107	2.5	3	1 mg.	Somewhat weak	-1.6
108	2.5	18	1 "	Few coughs, somewhat weak	-1.6
109	2.5	18	1 "	Negative	+0.55
110	1.25	0	1 "	Vigorous scratching	-1.9
111	1.25	0	1 "	Negative	-1.55
112	1.25	3	1 "	"	-0.75
113	1.25	3	1 "	"	-1.8
114	1.25	18	1 "	Spasms	-2.4
115	0.6	0	1 "	† 4 min.	
116	0.6	0	1 "	Convulsions, weak	-2.3
117	0.6	3	1 "	Spasms	-1.55
118	0.6	3	1 "	Negative	-1.25
119	0.6	3	1 "	"	0
120	0.6	18	1 "	† 4 min.	
121	0.3	0	1 "	Somewhat weak	-1.1
122	0.3	0	1 "	† 4 min.	
123	0.3	3	1 "	Dyspnea, somewhat weak	-1.45
124	0.3	3	1 "	Severe	-8.85
125	0.3	3	1 "	† 6 min.	
126	0.3	4	1 "	Negative	-0.9
127	0.15	0	1 "	† 4 min.	
128	0.15	0	1 "	† 4 "	
129	0.15	3	1 "	Very severe, almost dying	-2.4
130			0.5 "	† 5 min.	
131			0.5 "	† 6 "	
132			0.5 "	† 3 "	
133			0.5 "	† 4 "	
134			0.5 "	† 4 "	
135			0.5 "	† 3 "	
136			0.25 "	Slight to moderate	-3.0

tion by small quantities of antigen. On the first assumption one would expect protection to diminish with the elimination of the inhibiting substance from the blood stream. Actually the elimination takes place rather quickly since soon after the injection the urine is distinctly colored. Further evidence was gained from an examination of the color of the serum of guinea pigs after intravenous injection of the dye and from an estimation of the dye in the serum by inhibition of the precipitin reaction. From the few tests made it appeared that a considerable part of the azodye (about half) was already eliminated within the first hour after the injection.

On the other hand protection was still demonstrable on the day following the administration of the dye, and furthermore, in the experiments of Table VII there was no noticeable difference in the results whether the inhibiting substance was injected simultaneously with the antigen or 3 hours afterwards. Consequently one can conclude that the effect is not due simply to the presence in the circulation of the substances tested but to a desensitizing action upon the tissues. This view is corroborated by the observation that frequently the injection of homologous azodye into sensitized animals was followed by a significant drop in temperature and in a number of cases even by anaphylactic shock (17), but in some series of experiments this result could not be duplicated.

It is possible, however, that there are other instances in which protection is brought about by the same mechanism as inhibition *in vitro*. This is suggested by the results of Tillett, Avery, and Goebel, who noticed that their glucosides prevented shock only when injected just prior to the antigen, but not after an interval of 2 hours. The apparent discrepancy between their results and the present ones can probably be attributed to differences in the chemical nature of the substances used.

SUMMARY

Experiments with azoproteins containing stereo-chemical isomeric groups of *d*- and *l*-tartaric acid showed well marked specificity of the anaphylactic reaction to these antigens, in conformity with the results of precipitin tests.

Shock in these animals could be prevented by injection of azodye containing the specific groups. This phenomenon is ascribed to a desensitization.

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