ON THE SPECIFICITY OF SEROLOGICAL REACTIONS WITH SIMPLE CHEMICAL COMPOUNDS (INHIBITION REACTIONS)

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Whereas the usual serological reactions involve the use of high-molecular antigens of unknown constitution, a method has been described in a previous paper (1) which permits of the application of serological reactions to compounds of simple chemical composition. The method is based upon the possibility of partially synthesizing antigens (2, 3), by attaching to proteins chemical substances of simple structure as can be done by coupling with diazo compounds. Immune sera produced by injecting such "synthetic antigens" exhibit a specificity depending on the simple substances forming a part of the complex, particularly if the test antigen used contains a protein different from that used for immunization.

In ordinary precipitin tests, it has long been noticed that the reactions can be inhibited by addition of an excess of the antigen. Applying this observation to artificial complex antigens, it has been shown that the precipitin reactions of these substances are often inhibited specifically by the addition of a sufficient quantity of the same or a similar chemical substance to that used in building up the antigenic complex (1). These observations have since been confirmed by Klopstock and Selter (4), Avery and Goebel (5), and by ourselves.

The inhibition reactions with substances of known chemical structure can be used for the study of serological specificity in general, and in this regard they have certain advantages over the precipitin or complement fixation tests made with the full artificial antigens. Thus, in the first place it is not necessary to use substances which can be combined with proteins and, consequently, a greater variety of compounds can readily be subjected to the test. Furthermore an advantage may arise from the circumstance that the possible

influence of the protein part of the antigen, and of the mode in which the specific part is attached, are entirely excluded.

A particular application of the inhibition test has been suggested previously (1), namely the determining of the nature of the specific group in an antigen in cases in which this is not known. This plan has been followed in our laboratory by Wormall (6), who was able to demonstrate that in iodized protein the reacting group is the diiodotyrosine radical, since the precipitin reaction of the protein can be inhibited specifically by diiodotyrosine but not by ortho-iodophenol or potassium iodide. An essentially similar method has repeatedly been employed in recent studies on the specificity of ferments.

In the present paper, some additional results are discussed which were obtained in the course of serological studies on azoproteins.

Influence on the Specificity of the Position of Substituents in Aromatic Compounds

The significance of the position of substituents in the benzene ring for the specificity of precipitin reactions was established in tests (2) with immune sera, reacting on antigens containing various aromatic acids, *e.g.*, *o*-, *m*-, and *p*-aminobenzoic acid antigens.*

Similar conditions appeared to prevail in the inhibition tests. In fact, by means of an immune serum for *p*-aminobenzoic acid it was possible to differentiate various ortho- and para-monosubstituted benzoic acids, regardless of the nature of the substituent which was variously OH, CH_3, Br, Cl, or NO_2 (1). For, in all instances, solutions of the sodium salts of the *p*-acids were much more active than those of the *o*-acids, while the *m*-substituted compounds were intermediate in activity.

In order to obtain further information it seemed necessary to examine also immune sera for *o*- and *m*-substituted antigens which had not been studied sufficiently in the previous experiments. The antigens used for immunization were prepared by coupling diazotized *o*-, *m*-, and *p*-aminobenzoic acids with horse serum. For the technique of the prep-

* For the sake of brevity, the immune sera and antigens prepared with azoproteins will be designated by the compounds used for diazotization and subsequent coupling.

TABLE I

The following substances were used for the inhibition tests: (1) ortho-aminobenzoic acid; (2) meta-aminobenzoic acid; (3) para-aminobenzoic acid; (4) ortho-methylbenzoic acid; (5) meta-methylbenzoic acid; (6) para-methylbenzoic acid; (7) ortho-chlorobenzoic acid; (8) meta-chlorobenzoic acid; (9) para-chlorobenzoic acid; (10) ortho-bromobenzoic acid; (11) meta-bromobenzoic acid; (12) para-bromobenzoic acid; (13) ortho-oxybenzoic acid; (14) meta-oxybenzoic acid; (15) para-oxybenzoic acid; (16) ortho-nitrobenzoic acid; (17) meta-nitrobenzoic acid; (18) para-nitrobenzoic acid; (19) benzoic acid.

The tests with the meta-aminobenzoic acid immune serum No. 2 were made at 37°, the other tests at room temperature.

Immune serum for	Serum No.	Reading after	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	C
Ortho-aminobenzoic acid	1	15 min.	0	0	±	0	0	0	0	0	0	0	0	0	0	0	±	0	0	0	0	+
		1 hr.	0	tr.	+	0	0	f. tr.	0	0	0	0	0	0	0	tr.	+	0	0	tr.	0	+±
		2 hrs.	0	±	+	0	0	tr.	0	0	f. tr.	0	0	0	0	±	+	0	0	tr.	0	+±
		3 hrs.	0	±	+	0	0	±	0	0	f. tr.	0	0	0	0	+	+	0	0	±	0	+±
		Night in ice box	±	+	+±	±	±	+	tr.	tr.	∓	tr.	tr.	±	tr.	+	+±	tr.	tr.	+	±	+±
Meta-aminobenzoic acid	2	15 min.	0	0	±	±	0	0	f. tr.	0	0	tr.	0	0	f. tr.	tr.	±	tr.	0	0	0	+±
		1 hr.	tr.	0	+	+	0	tr.	±	0	0	±	0	0	tr.	tr.	+±	∓	0	f. tr.	0	++±
		2 hrs.	±	0	+	+	0	tr.	∓	0	0	∓	0	tr.	tr.	tr.	+±	∓	0	tr.	0	+++
		Night in ice box	±	tr.	+±	+	f. tr.	±	+	0	±	∓	f. tr.	∓	±	∓	+±	∓	0	±	tr.	+++
Meta-aminobenzoic acid	3	15 min.	tr.	f. tr.	±	±	0	tr.	tr.	0	f. tr.	tr.	0	0	0	f. tr.	±	tr.	0	tr.	0	+±
		1 hr.	∓	tr.	+	+	0	∓	∓	0	±	±	0	tr.	tr.	±	+	∓	0	∓	tr.	++
		2 hrs.	∓	±	+	+	0	+	+	0	∓	+	0	±	tr.	∓	+	+	0	+	±	++
		Night in ice box	+	+	+	+	∓	+	+	∓	+	+	±	+	+	+	+±	+±	±	+±	∓	++
Para-aminobenzoic acid	4	15 min.	tr.	tr.	0	±	0	0	tr.	0	0	±	0	0	f. tr.	±	0	±	0	0	f. tr.	+
		1 hr.	tr.	±	0	∓	f. tr.	0	∓	f. tr.	0	+	0	0	tr.	∓	0	+	0	0	tr.	+±
		2 hrs.	±	∓	0	+	tr.	f. tr.	+	tr.	0	+	f. tr.	0	tr.	∓	0	+	f. tr.	0	tr.	+±
		3 hrs.	±	∓	0	+	tr.	f. tr.	+	tr.	0	+	f. tr.	0	±	∓	f. tr.	+	f. tr.	0	±	+±
		Night in ice box	±	∓	tr.	+	±	tr.	+	±	tr.	+	tr.	tr.	±	∓	tr.	+	tr.	tr.	±	++

aration of the antigens, and the immunization, we refer to previous communications (1, 7).

For the inhibition tests 1 millimol of each substance to be tested was dissolved in water by the aid of sodium hydroxide, and the solution made neutral to litmus and brought up to a volume of 10 cc. Unless otherwise stated, in each test 0.05 cc. of this solution was mixed with 0.2 cc. of the diluted antigen (made with chicken serum) previous to the addition of the immune serum. The control tube (C) contained only antigen and immune serum. The dilutions of the antigens are in terms of a 5 per cent stock solution. The intensity of the precipitin reaction is indicated as follows: 0, f. tr. (faint trace), tr. (trace), tr. (strong trace), ±, ±+, +, +±, etc.

The results in Table I are what would be expected from former findings, that is, comparing *o*- and *p*-immune sera and the *o*- and *p*-substances, the inhibition was always more intense with the "homologous" substance. Also the inhibition of the precipitation by the *m*-aminobenzoic immune sera was in general strongest with meta-substituted compounds, though in the case of the oxybenzoic acids the inhibition effect of the ortho-substance was somewhat greater than that of the meta-substituted acid. On comparing *o*- and *p*-derivatives of benzoic acid, it was seen moreover that substitution in the "heterologous" position often diminishes considerably the inhibition reaction as compared not only with the "homologous" substance, but also with the non-substituted benzoic acid.

An influence of greater or lesser strength of the acids seems to be excluded as the cause of these effects, because the regularities observed are of the same sort for substituted acids which differ widely in their strength, and also because of the results with the *m*-substituted substances. As suggested previously (1, 2), the most probable explanation is that the outcome of the reactions in the cases under consideration depends on the steric correspondence in the position of the substituents in the immunizing antigens and the compounds employed for inhibition, even though the substances examined differ in their composition from the specifically binding groupings in the antigens.

A relationship of the observations described to the phenomenon of ferment specificity is indicated by the findings of Waldschmidt-Leitz and Balls (8), who reported that of the isomer (chloroacetylamino) benzoic acids only the meta-compound is hydrolized by pancreatic

carboxypolypeptidase, a fact which the authors also explain on the basis of steric configuration. Other similar instances are the inhibition of the action of tyrosinase by aromatic acids (9), and the oxidation of various substrates by this enzyme (10). It is noteworthy that a specificity depending on the position of aromatic substituents has been observed recently in the skin reactions of a patient sensitive to resorcinol (meta-dihydroxybenzene) by Nathan and Stern (11). This patient did not react to either ortho- or para-dihydroxybenzene, nor to the methyl ethers of resorcinol, nor to phenol or pyrogallol.

Not all sera are equally well suited for the demonstration of the inhibitory effects. Thus with some sera which fell off after long storage and also with some fresh sera, the inhibition by benzoic acid was weaker than usual and the regularities described were blurred. In some such instances it was possible to increase the inhibition by heating the immune sera for 1 hour at 55°C. or by setting up the tests at that temperature.

Inhibition Effects by Fatty and Cyclic Acids

In the tests described previously (1), the reaction of a serum for *p*-aminobenzoic acid antigen was found to be inhibited by a variety of aromatic acids, *i.e.* benzoic acid and substituted benzoic acids, but not distinctly by several aliphatic acids tested. Since benzoic acid caused practically no interference with the precipitin reaction by sera for *p*-arsanilic acid or *m*-aminobenzenesulfonic acid—which, in turn, are inhibited by aromatic arsinic or sulfonic acids—it is plain that the effect produced by benzoic acid depends on the constitution of the substance containing a carboxyl group bound to the benzene ring, a structure which corresponds to that of the binding group in the antigen. In recent experiments it was, however, found that aliphatic carbonic acids, especially the higher ones such as caproic and heptylic acids, also inhibit the reaction of immune sera for aminobenzoic acids.

As Table II shows, the effect increased with the length of the chain and was most marked with the caproic and heptylic acids. Acids with much longer chains could not be properly tested on account of the turbidity of the neutral solutions, but from a test with caprylic acid it would seem that the action may be stronger with the higher members of the series. Consequently it is possible that the phenomena, at least in part, are due to the physicochemical properties of high fatty

TABLE II

The following substances were used for the inhibition tests: (1) benzoic acid; (2) cyclohexane carboxylic acid; (3) acetic acid; (4) propionic acid; (5) normal butyric acid; (6) isobutyric acid; (7) normal valeric acid; (8) isovaleric acid; (9) methyl-ethylacetic acid; (10) normal caproic acid; (11) isocaproic acid; (12) diethylacetic acid; (13) normal heptylic acid.

Immune serum for	Serum No.	Reading after	1	2	3	4	5	6	7	8	9	10	11	12	13	C
Ortho-aminobenzoic acid	5	15 min.	0	0	+±	+±	+	+	±	±	±	tr.	±	+	±	++
		1 hr.	0	±	++	++	+±	+±	+	+±	+±	+	+	+±	+	++±
Para-aminobenzoic acid	6	5 min.	0	0	+	±	±	+	±	±	±	tr.	±	+	f. tr.	+±
		1 hr.	0	±	++	++	+±	++	+±	+±	+±	+	+±	++	+	+++

TABLE III

The following substances were used for the inhibition tests: (1) benzoic acid; (2) α-thiophene carboxylic acid; (3) nicotinic acid; (4) cinchonic acid; (5) furan carboxylic acid; (6) picolinic acid; (7) cyclohexane carboxylic acid; (8) α-pyrrole carboxylic acid; (9) α-naphtoic acid; (10) β-naphtoic acid; (11) acetic acid; (12) normal caproic acid; (13) normal caprylic acid.

Immune serum for	Serum No.	Reading for	1	2	3	4	5	6	7	8	9	10	11	12	13	C
Ortho-aminobenzoic acid	1	15 min.	0	0	tr.	0	tr.	±	f. tr.	tr.	0	0	+	tr.	f. tr.	+
		1 hr.	0	0	±	0	±	±	tr.	±	0	0	+	±	tr.	+
		2 hrs.	0	f. tr.	+	f. tr.	+	+	±	+	0	f. tr.	+±	+	±	+±
Meta-aminobenzoic acid	2	15 min.	0	f. tr.	tr.	tr.	tr.	±	±	±	0	0	+	±	±	+
		1 hr.	f. tr.	tr.	tr.	tr.	±	+	±	+	0	0	+±	+	±	+±
		2 hrs.	tr.	±	±	+	±	+±	+±	+±	0	0	++	+±	+±	++
Meta-aminobenzoic acid	3	15 min.	0	f. tr.	tr.	±	±	±	±	±	0	f. tr.	+±	+	±	+±
		1 hr.	tr.	tr.	±	+	+	+	+	+	f. tr.	tr.	++	+	+	++
		2 hrs.	±	±	+	+±	+±	+±	+±	+±	tr.	±	++±	+±	+±	++±
Para-aminobenzoic acid	4	15 min.	f. tr.	0	tr.	f. tr.	tr.	±	tr.	±	f. tr.	0	+	±	±	+
		1 hr.	tr.	f. tr.	±	tr.	±	±	±	±	tr.	0	+	±	±	+
		2 hrs.	tr.	tr.	±	±	±	+	+	+	±	0	+±	+	+	+±

acids. In this connection it should be mentioned that salts of cholic and desoxycholic acids appear to inhibit precipitin reactions in general. On the other hand, the chemical structure, particularly the presence of the carboxyl group, must play a part, since other precipitin reactions, *e.g.* that of an immune serum for arsanilic acid, are only slightly influenced by caproic or heptylic (or benzoic) acids. The fact that these acids have very little effect on precipitin reactions of proteins or carbohydrates, such as horse serum or a precipitable substance of pneumococci, may be attributable to the more intricate constitution of the specifically reacting groups in these antigens.

Table III illustrates the inhibitory action of some cyclic acids on the precipitation by *o*- and *p*-aminobenzoic acid immune sera.

The tests show that not only benzoic acid but also other cyclic acids exert inhibiting effects. Intense inhibition was caused by α-thiophene carboxylic acid and α- and β-naphtoic acids, the result with the former being in good agreement with the close chemical similarity between α-thiophene carboxylic acid and benzoic acid.

Inhibition Reactions of Pronounced Specificity

The experiments reported in the present and a previous communication show that the reactions of certain immune sera can be inhibited by numerous substances, provided these are chemically somewhat similar to the reacting group of the antigen used. Such is the case when the groupings responsible for the specific precipitation have a simple structure, as for example in the antigens prepared with aminobenzoic acids. Extending the well known simile of E. Fischer, these instances may be compared to the unlocking of a simple lock by various keys or of various locks by a simple key. Accordingly it appears that the inhibitory effect can result from weak affinities which would not be sufficient for causing specific precipitation, *e.g.*, a serum for para-aminobenzoic acid would be inhibited to a certain degree by ortho-aminobenzoic acid but would not precipitate test antigens built up from the latter substance.

It has already been suggested that the range of reactivity is narrowed in cases in which either the antigen or the inhibiting substance is of somewhat more complex chemical structure. Inhibition reactions with azodyes (1) are instances of the sort. Since this view

has not been checked in any case by tests with numerous compounds of widely varied composition, and on account of the results reported in the preceding section, it seemed desirable to verify it by experiments carried out on a larger scale. To this end, two antigens and the corresponding immune sera, studied previously (12, 13), were selected with specific groups of more complicated constitution than those used in our first studies, namely two azoproteins, prepared from *l*-phenyl (para-aminobenzoylamino) acetic acid, and *l*-para-aminotartranilic acid. For the inhibition tests a large number of organic acids of widely varying chemical constitution were taken.

The following substances were used for the inhibition tests: (1) acetic acid; (2) normal butyric acid; (3) normal valeric acid; (4) isovaleric acid; (5) normal caproic acid; (6) isocaproic acid; (7) diethylacetic acid; (8) normal heptylic acid; (9) chloroacetic acid; (10) bromoacetic acid; (11) cyanacetic acid; (12) trichloroacetic acid; (13) β-iodopropionic acid; (14) lactic acid; (15) levulinic acid; (16) aminoisovaleric acid; (17) acrylic acid; (18) α-crotonic acid; (19) *d, l*-bromosuccinic acid; (20) citric acid; (21) fumaric acid; (22) glycocoll; (23) *d, l*-alanin; (24) phenylglycine; (25) *d, l*-phenylalanin; (26) *d, l*-leucine; (27) histidine; (28) benzoylglycine; (29) *d, l*-benzoylalanine; (30) *l*-asparaginic acid; (31) *l*-glutaminic acid; (32) camphoric acid; (33) cyclohexane carboxylic acid; (34) benzoic acid; (35) ortho-aminobenzoic acid; (36) meta-aminobenzoic acid; (37) para-aminobenzoic acid; (38) 4-chloro-3-aminobenzoic acid; (39) ortho-chlorobenzoic acid; (40) meta-chlorobenzoic acid; (41) para-chlorobenzoic acid; (42) ortho-bromobenzoic acid; (43) meta-bromobenzoic acid; (44) para-bromobenzoic acid; (45) ortho-nitrobenzoic acid; (46) meta-nitrobenzoic acid; (47) para-nitrobenzoic acid; (48) ortho-oxybenzoic acid; (49) meta-oxybenzoic acid; (50) para-oxybenzoic acid; (51) ortho-methylbenzoic acid; (52) meta-methylbenzoic acid; (53) para-methylbenzoic acid; (54) 2, 4, dinitrobenzoic acid; (55) 2, 4, 6, trinitrobenzoic acid; (56) anisic acid; (57) 1, 2, 5, dioxybenzoic acid; (58) 1, 2, 4, dioxybenzoic acid; (59) benzolsulfonic acid; (60) ortho-aminobenzolsulfonic acid; (61) meta-aminobenzolsulfonic acid; (62) para-aminobenzolsulfonic acid; (63) sulfosalicylic acid; (64) para-toluidinsulfonic acid; (65) para-azoxybenzoic acid; (66) phenylacetic acid; (67) mandelic acid; (68) benzilic acid; (69) vanillic acid; (70) cinnamic acid; (71) hydrocinnamic acid; (72) ortho-aminocinnamic acid; (73) meta-aminocinnamic acid; (74) para-aminocinnamic acid; (75) ortho-nitrocinnamic acid; (76) meta-nitrocinnamic acid; (77) para-nitrocinnamic acid; (78) ortho-cumaric acid; (79) phthalic acid; (80) isophthalic acid; (81) terephthalic acid; (82) α-thiophene carboxylic acid; (83) furan carboxylic acid; (84) meconic acid; (85) α-naphtoic acid; (86) β-naphtoic acid; (87) α-oxynaphtoic acid; (88) β-oxynaphtoic acid; (89) naphtionic acid; (90) α-pyrrole carboxylic acid; (91) picolinic acid; (92) nicotinic acid; (93) cinchonic acid.

For the tests 0.05 cc. of a neutral solution of the substance containing 0.5 millimol in 10 cc. water were used.

TABLE IV

Substance No.	Immune serum for levo-phenyl-(para-amino-benzoylamino) acetic acid		Immune serum for levo-para-amino-tartranilic acid		Substance No.	Immune serum for levo-phenyl (para-amino-benzoylamino) acetic acid		Immune serum for levo-para-amino-tartranilic acid	
1	+	++	+±	++±	48	+	++	+±	++
2	+	++	+±	++±	49	+	++	+±	++±
3	+	++	+±	++	50	+	++	+±	++
4	+	++	+±	++±	51	+	++	+±	++
5	+	++	+±	++±	52	+	++	+±	++
6	+	++	+±	++±	53	+	++	+±	++±
7	+	++	+±	++	54	±	++	+±	++
8	+	++	+±	++±	55	±	+±	+±	++±
9	+	++	+±	++	56	+	++	+±	++
10	+	++	+±	++	57	+	++	+±	++
11	+	++	+±	++	58	+	++	+±	++
12	+	++	+±	++	59	+	++	+±	++±
13	+	++	+±	++±	60	+	++	+±	++
14	+	++	+±	++	61	+	++	+±	++
15	+	++	+±	++	62	+	++	+±	++
16	+	++	+±	++	63	+	++	+±	++±
17	+	++	+±	++±	64	+	++	+±	++
18	+	++	+±	++	65	+	++	+±	++
19	+	++	+±	++	66	±	++	+±	++
20	+	++	+	++	67	±	++	+	++
21	+	++	+±	++	68	±	++	+±	++
22	+	++	+±	++	69	+	++	+±	++±
23	+	++	+±	++	70	±	++	+±	++
24	±	++	+±	++	71	±	++	+±	++
25	+	++	+±	++	72	+	++	+±	++±
26	+	++	+±	++	73	+	++	+±	++±
27	+	++	+±	++	74	+	++	+±	++
28	±	++	+±	++	75	tr.	+	+±	++±
29	±	++	+±	++±	76	±	++	+±	++±
30	+	++	+±	++±	77	±	++	+±	++
31	+	++	+±	++±	78	+	++	+±	++
32	+	++	+±	++	79	+	++	+±	++±
33	±	++	+±	++±	80	+	++	+±	++±
34	+	++	+±	++±	81	+	++	+±	++
35	+	++	+±	++	82	±	++	+±	++±
36	+	++	+±	++	83	+	++	+±	++±
37	+	++	+±	++	84	+	++	+±	++±
38	+	++	+±	++	85	±	++	+±	++
39	+	++	+±	++±	86	±	+±	+±	++
40	+	++	+±	++	87	+	++	+±	++
41	+	++	+±	++±	88	+	++	+±	++
42	+	++	+±	++	89	+	++	+±	++
43	±	+±	+±	++±	90	+	++	+±	++
44	+	++	+±	++±	91	+	++	+	++
45	+	++	+±	++±	92	±	++	+±	++
46	+	++	+±	++	93	±	+±	+±	++
47	+	++	+±	++	Hom. subst.	0	±	0	0
					Control	+	++	+±	++

In the table, the first column gives the readings after 2 hours at room temperature for each of the two immune sera, the second column those after standing overnight in the ice box "Hom. subst." stands for "homologous substance," namely: levo-phenyl (para-aminobenzoylamino) acetic acid, or levo-para-aminotartranilic acid.

It is seen from Table IV that in the tests with the first immune serum there occurred some inhibitions with non-homologous substances which, with one exception, disappeared or became very weak on longer standing. The homologous substance gave the strongest effect. With the immune serum for aminotartranilic acid only three heterologous substances showed relatively weak inhibition in the first reading, whereas the later reading demonstrated a perfectly specific reaction of the homologous substance. The inhibition with the homologous substance was complete also with one-half of the quantity used in the experiment presented and distinct with one-eighth of that quantity.

It may be concluded, therefore, that under appropriate conditions the inhibition tests exhibit a high degree of specificity, comparable to that of the common serological phenomena. Thus, the peculiar specificity displayed in serum reactions is not limited to high molecular colloids, but can be demonstrated as well with relatively simple crystallized substances, and is largely independent of the molecular size.

SUMMARY

Experiments are described which confirm the result that the specificity of inhibitory reactions involving substituted aromatic acids is decidedly influenced by the position of the substituent.

When antigens with specific groups of very simple constitution are used for the tests, inhibiting effects are obtained also with substances distantly related to those determining the reactivity of the antigens. On the other hand, if antigens are built up from protein and chemical compounds of somewhat higher complexity, the specificity of the inhibition reactions with synthetic crystallized substances is of the same order as that of the usual serum reactions; in other words, it is possible to distinguish such compounds by serological tests as readily as proteins can be differentiated with the aid of precipitating sera.

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