ON CROSS REACTIONS OF IMMUNE SERA TO AZOPROTEINS

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(Received for publication, November 23, 1935)

The overlapping reactions commonly observed in serological tests are ascribed by most authors to multiple antibodies formed as a result of the presence of several substances or distinct specific groups in the immunizing antigen. Yet studies on azoprotein immune sera have shown that antibodies corresponding to a particular compound regularly react with other substances which are sufficiently related in chemical structure (1, 2). Thus cross reactions in general can be understood on this principle alone without postulating a multiplicity of antibodies. However, this explanation does not cover the observations made when immune sera are partially exhausted by heterologous antigens.

It is unnecessary to cite examples of the well known and widely used fact that immune sera for bacteria and blood cells after treatment with a heterologous antigen (sensitive to its action) still react with the homologous antigen but no longer with that used for exhaustion. This method does not succeed as readily and regularly with precipitin sera for proteins (3). Yet in a number of experiments such sera could be made highly specific by fractional precipitation with heterologous antigen which reveals the presence of several antibodies. For instance, Hooker and Boyd (4) found that a precipitin for chicken serum acting also on duck serum, after removal of the precipitate formed on addition of the latter, still precipitated chicken serum intensely, but no longer duck serum. The assumption made here and in similar instances that the sera contain several antibodies directed toward different determinant groups in the protein has not been proved by chemical evidence. In order to investigate this question experiments were carried out with precipitins for artificially conjugated antigens

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in which the chemical constitution of the reacting groups is known. For absorption, to avoid complications which may arise from the presence of an excess of heterologous antigen remaining in solution, insoluble antigens were chiefly used, namely blood stromata coupled with diazonium compounds (azostromata).

The essential result, *i.e.* the demonstration of the presence of multiple antibodies in an immune serum formed in response to an individual specific structure, has already been communicated briefly (2, 5). In another way, namely by the quantitative study of precipitin reactions, Heidelberger and Kendall (6) have arrived at similar conclusions.

Technique

Preparation of Antigens.—The azoproteins used for immunization were made as described previously (7, 8) using either horse serum or horse serum globulin as the protein component; about 6 millimols of the substance were coupled to 100 cc. of serum (or 7 gm. of protein) except in the case of para-aminosuberanilic acid in which twice the amount of substance was used. For purification the antigens were dissolved in water by means of dilute sodium hydroxide, and precipitated by an equal volume of alcohol and the requisite amount of acid. The precipitates were separated by centrifuging, washed several times with saline and were dissolved in saline with the aid of dilute sodium hydroxide, avoiding a large excess, and the solutions made neutral to litmus.

Tests.—The antigens used for the tests were prepared from chicken serum or casein as described previously (7, 8). The dilutions of the test antigens given in the tables are in terms of a 5 per cent stock solution. The intensity of the reactions is indicated as follows: 0, f. tr. (faint trace), tr. (trace), $\underline{\text{tr.}}$ (strong trace), ±, ∓, +, +±, ++, etc.

Coupling of Stromata with Diazonium Compounds.—For the preparation of stromata the method described by Sachs (9) was followed in the main. The blood corpuscles freed from serum proteins by repeated washings with saline were brought to twice the original blood volume with saline and kept at 56–60° for 40 minutes. Five volumes of distilled water were added and after thorough shaking and standing at room temperature for ½ hour enough 10 per cent salt solution was added to bring the salt concentration to 1 per cent. The stromata were separated from the liquid by centrifugalization and were washed repeatedly with saline until the liquid was free from hemoglobin. For coupling 0.1 millimol of the amino compound dissolved in 2 cc. of water and 0.3 cc. of N HCl was diazotized with 1 cc. N/10 sodium nitrite, and ⅔ of the solution was added to a suspension of 50 mg. of stromata in 5 cc. water and 0.6 cc. N sodium carbonate. After allowing the mixture to stand at 0–5° for ½ hour, the remainder of the diazotized substance and 0.3 cc. of N sodium carbonate were added and the coupling

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allowed to proceed for another ½ hour (at 0–5°). After the addition of normal hydrochloric acid to weakly acid reaction to Congo red, the azostromata were centrifuged and washed several times with saline, the suspension made weakly alkaline to litmus by addition of normal sodium carbonate and the stromata washed with neutral saline until the supernatant liquids were perfectly colorless.

The azostromata used for absorption were prepared from rabbit blood, those for immunization from horse blood.

Immunization.—Rabbits were given daily intravenous injections of 2 cc. of the antigen solutions containing 10 mg. of azoprotein in 1 cc. Two or more courses of 6 daily injections were given at intervals of 1 week and the sera were tested 7 days after the last injection. It was found, furthermore, that satisfactory immune sera could be obtained by injecting suspensions of azostromata prepared by coupling the diazotized substances to stromata as described. Three to four courses of intravenous injections (2 cc.) were given as above of suspensions containing 2.5 mg. of azostromata in 1 cc. These sera gave precipitin reactions with the test antigens like sera produced with azoproteins; besides they were found to contain agglutinins and lysins for the blood from which the stromata were prepared. Absorptions with these blood corpuscles did not remove the precipitating antibodies.

Absorption Experiments.—Azostromata were packed by centrifuging and the sediment was mixed with concentrated or dilute immune serum by stirring. The mixture was allowed to remain at room temperature for 2 hours with occasional stirring, and the sediment was removed by centrifuging. Homologous and other specifically related immune sera produced distinct agglutination of the azostromata suspensions. The insolubility of the azostromata was demonstrated by testing the supernatant fluids of centrifuged suspensions and absorbed fluids with immune serum. In such tests no precipitation was observed, neither did the liquids give any yellow color on addition of alkali as is the case with even very dilute solutions of azoproteins.

Elution of Precipitins.—For example, 3 cc. of an immune serum (diluted 1:2 with saline) were absorbed with 2 mg. of azostromata. After centrifuging and washing twice with saline solution the stromata were suspended in 0.8 cc. of saline solution and 0.1 cc. of N/10 acetic acid was added. After 5 minutes the solution was freed from stromata by centrifuging and brought to pH 7.2 by addition of N/10 sodium hydroxide. The relative quantities of immune serum, azostromata and volume of eluent had to be determined for each serum.

Absorption Experiments

When an immune serum is treated with a properly chosen amount of one of several related antigens, giving reactions of different intensity, a fluid may be obtained which no longer reacts with the antigen used for exhaustion but still with those that have greater affinity for the immune serum, particularly with the homologous antigen. This

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is illustrated in the tabulated experiments made with an azoprotein immune serum (Table I).

These effects can be explained by the diminution in antibody content without presuming that the immune serum contains more than one antibody. However, there are other observations in which such an

TABLE I

1.5 cc. of suberanilic acid (7) immune serum (No. 1) (diluted 1:4 with saline) were absorbed with 1 mg. of glutaranilic acid azostromata for 2 hours at room temperature; in the same way absorptions were made with adipanilic, pimelanilic and suberanilic acid azostromata. For the tests 0.2 cc. of the absorbed fluids was mixed with 0.04 cc. of the test antigen (dilution 1:125 of a 5 per cent solution).

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Suberanilic acid immune serum absorbed with azostromata made from	Azoprotein made from casein and			
	p-Aminoglutar-anilic acid B*-$(CH_2)_3$-COOH	*p*-Aminoadip-anilic acid B-$(CH_2)_4$-COOH	*p*-Aminopimel-anilic acid B-$(CH_2)_5$-COOH	*p*-Aminosuber-anilic acid B-$(CH_2)_6$-COOH
p-Aminoglutaranilic acid	0 0	tr. tr.	+ +	++ ++±
p-Aminoadipanilic acid	0 0	0 0	tr. ±	+± ++
p-Aminopimelanilic acid	0 0	0 0	0 0	tr. ±
p-Aminosuberanilic acid	0 0	0 0	0 0	0 0
Unabsorbed immune serum diluted 1:4 with saline	± +	+± ++	++ ++±	++± +++

* B– represents $NH_2C_6H_4NHCO$.

explanation is excluded since the immune serum gave reactions of much greater specificity after partial absorption than on mere dilution. An example is given in Table II.

By cross absorption, using two heterologous azoproteins, the immune sera could be made specific for either antigen. Such experiments were performed, for example, with immune sera for *m*-aminobenzenesulfonic

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TABLE II

Suberanilic acid immune serum (No. 2) (diluted 1:2 with saline) was absorbed with adipanilic acid azostromata as described in Table I. Similarly, a metanilic acid (1) immune serum (No. 1) was absorbed with *o*-aminobenzenesulfonic acid azostromata. The specificity of the absorbed fluids was compared with that of unabsorbed immune serum. From the immune serum diluted 1:2 with saline higher dilutions were made with saline and with normal rabbit serum (diluted 1:2), the object of using normal rabbit serum being to maintain a constant protein content.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Suberanilic acid immune serum	Azoproteins made from casein and		Metanilic acid immune serum	Azoproteins made from chicken serum and	
	p-Amino-adip-anilic acid	*p*-Amino-suber-anilic acid		*o*-Amino-benzene-sulfonic acid	Metanilic acid
Diluted 1:2 with saline, absorbed with adipanilic acid azostromata	0 0	++± +++	Diluted 1:2 with saline, absorbed with *o*-aminobenzenesulfonic acid azostromata	0 0	+± ++±
Unabsorbed, diluted 1:2 with saline	++ ++±	+++± ++++	Unabsorbed, diluted 1:2 with saline	++ ++	+++ +++
Unabsorbed, diluted 1:4 with saline	+ +±	++ ++±	Unabsorbed, diluted 1:4 with saline	± +±	+± ++±
Unabsorbed, diluted 1:3 with normal rabbit serum	+± ++±	++± +++	Unabsorbed, diluted 1:4 with normal rabbit serum	± +±	+± ++±
Unabsorbed, diluted 1:4 with normal rabbit serum	+ ++	++ ++±	Unabsorbed, diluted 1:5 with normal rabbit serum	tr. +	+ ++
Unabsorbed, diluted 1:6 with normal rabbit serum	± +	+ +±	Unabsorbed, diluted 1:6 with normal rabbit serum	tr. ±	+ +±

(metanilic) acid which were found to give cross reactions[1] with chemically related antigens as shown in Table III.

[1] Some of the immune sera gave definitely weaker cross reactions which were seen only after the test mixtures had been kept overnight in the ice box.

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As will be seen from Table IV an immune serum for metanilic acid after absorption with *o*-aminobenzenesulfonic acid azostromata still precipitated *m*-aminophenylarsenic acid antigen. An analogous result was obtained when the experiment was set up in reversed order.

Equally striking was the result when more than two antigens were used (Table V). Here the supernatant fluids resulting from absorption with an heterologous azoprotein reacted on all other antigens while that used for absorption was not precipitated, or only very

TABLE III

2 drops of immune serum (No. 1) for *m*-aminobenzenesulfonic acid were added to 0.2 cc. of the antigens, diluted as indicated in the table.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Antigens made from chicken serum and	Position of the substituents						
	o-NH_2, R*			*m*-NH_2, R	*p*-NH_2, R		
	Dilution			Dilution	Dilution		
	1:100	1:500	1:2500	1:500	1:100	1:500	1:2500
Aminobenzenesulfonic acid		+± +±		++± +++		tr. ±	
Aminophenylarsenic acid	0 0	0 0	0 0	+ +	0 0	0 0	0 0
Aminobenzoic acid	0 0	0 0	0 0	± +	0 0	0 0	0 0

* R denotes the groups SO_3H, AsO_3H_2, COOH.

slightly; the homologous antigen, however, upon performing repeated absorptions removed the reactions with heterologous antigens first, finally the homologous one. The effects were not equally pronounced with individual metanilic immune sera but were always noticeable, depending, however, on the amount of absorbing material, since in general by repeated exhaustion with heterologous antigens all precipitin reactions were progressively diminished. With some antigens[2]

[2] Slight diminution of antibodies due to specific affinities is not easily demonstrable since treatment with any sort of azostromata will cause some reduction in antibody content.

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TABLE IV

Metanilic acid immune serum (No. 1) was absorbed with *o*-aminobenzenesulfonic acid and with *m*-aminophenylarsenic acid azostromata using 2 mg. of stromata and 1.5 cc. of undiluted immune serum for each absorption. The tests were made as described in Table I.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Metanilic acid immune serum absorbed with azostromata made from	Azoproteins made from chicken serum and		
	o-Aminobenzene-sulfonic acid	Metanilic acid	*m*-Aminophenylarsenic acid
o-Aminobenzenesulfonic acid	0 0	++ ++±	+ +±
m-Aminophenylarsenic acid	+± ++±	++± +++	0 0

TABLE V

Metanilic acid immune serum (No. 2) was absorbed with azostromata prepared from *o*-aminobenzenesulfonic, *m*-aminophenylarsenic and *m*-aminobenzoic acid azostromata, using 0.6 mg. azostromata for 1.5 cc. undiluted immune serum. The tests were made as described in Table I.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Metanilic acid immune serum absorbed with azostromata made from	Azoproteins made from chicken serum and			
	o-Aminobenzene-sulfonic acid	Metanilic acid	*m*-Aminophenyl-arsenic acid	*m*-Aminobenzoic acid
o-Aminobenzenesul-fonic acid	0 0	++± +++±	± ±	+ +
m-Aminophenylarsenic acid	+± ++	+++ ++++	0 0	+ +±
m-Aminobenzoic acid	+± ++	+++ ++++	± ±	0 f. tr.
Unabsorbed immune serum	++ +++	+++± ++++	+ ++	+± ++±

(*m*-arsanilic acid, *m*-aminobenzoic acid), reacting weakly, repeated absorption produced no significant diminution of the homologous reaction.

In the experiments presented the adsorption was carried out with

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TABLE VI

Undiluted suberanilic immune serum (No. 3) was absorbed with adipanilic acid and sebacanilic acid azostromata (3 and 2 mg. respectively). The quantities used are indicated in the table. The tests were made as described in Table I.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

1 cc. of suberanilic acid immune serum absorbed with azostromata made from	Azoproteins made from casein and		
	p-Aminoadipanilic acid	*p*-Aminosuberanilic acid	*p*-Aminosebacanilic acid
p-Aminoadipanilic acid	0 0	++± +++±	+± ++
p-Aminosebacanilic acid	+ +±	+++ ++++	0 0
Unabsorbed immune serum	+++ +++±	++++ ++++	+++ +++±

TABLE VII

Glycyl-leucine (GL) immune serum (No. 1) (diluted 1:3 with saline) was absorbed with glycyl-glycine (GG), *d,l*-leucyl-*d,l*-leucine (LL) and *d,l*-leucine (L) azostromata. In each case 1 mg. of azostromata was used for 1.5 cc. of diluted immune serum. The tests were made as described in Table I.

Readings were taken after 3 hours at room temperature (first line) and after standing overnight in the ice box (second line).

GL immune serum absorbed with azostromata made from	Azoproteins made from chicken serum and			
	GG	GL	LL	L
GG	0 0	+++ +++	+± ++±	++ ++±
LL	+ +	+++ +++	0 0	± +
L	± +	+++ +++	f. tr. tr.	0 0
Immune serum diluted 1:3, unabsorbed	+ +±	+++± +++±	++ ++±	++± +++

blood stromata coupled with the respective diazonium compounds, but conformable results were obtained by partial precipitation with soluble azoproteins.

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Similar observations were made with immune sera for suberanilic (7) acid and amino-benzoyl-glycyl-leucine (8) (Tables VI and VII). In some other cases, for example in the cross reactions of aminophenylarsenic antisera on antigens made from other phenylarsenic acids, no definite effects like those described above were obtained.

It should be mentioned incidentally that in azoprotein sera there are often, in addition to the antibodies specific for the azocomponent, others separable by absorption, which act on the original proteins (Heidelberger and Kendall (10)). The sera may also contain antibodies which precipitate azoantigens made from the same protein as the immunizing antigen and various diazonium compounds.

Elution of Absorbed Precipitins

After absorption of azoprotein immune sera with azostromata, as in the above experiments, precipitins can easily be liberated by treating the stromata with dilute acetic acid. These solutions, presumably, consist largely of antibody protein and have a low nitrogen content.

For instance, 6 cc. of metanilic acid immune serum were exhausted with 8 mg. metanilic acid azostromata; after several washings with saline, antibodies were liberated as described. The solution (6 cc.) which had half the potency of the original serum, titrated by diluting with normal rabbit serum, contained approximately 0.5 mg. protein per cc., indicating that the antibody protein in the serum amounted to about 0.7 per cent of the whole protein content, supposing that no significant non-specific adsorption of proteins had taken place.

In several cases the results obtained with antibodies separated by elution were in agreement with those already described, whereas in other instances (*e.g.* metanilic acid immune serum absorbed with *o*-aminobenzenesulfonic acid stromata) the purified antibody solutions obtained from a serum previously made specific by absorption with an heterologous stroma antigen again gave strong precipitation with the corresponding heterologous azoprotein, an apparent inconsistency. It was found, however, that the purified antibody solutions had more or less the tendency to precipitate non-specifically even azoproteins unrelated to the homologous antigen, probably owing to instability of the solutions. This difficulty could be overcome through increasing the protein concentration by the addition of normal rabbit serum; but since dilution with normal serum weakens the reactions, this method has so far not proved to be fully satisfactory for specificity tests.

Inhibition Tests

The specificity of the antibodies responsible for cross reactions of azoprotein sera was examined further by means of inhibition tests.

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TABLE VIII

o-Aminobenzenesulfonic acid (1), metanilic acid (2), *m*-aminophenylarsenic acid (3) and *m*-aminobenzoic acid (4) were dissolved in water by the aid of sodium hydroxide and the solutions were made neutral to litmus and brought to the required volume. In test I, 0.1 cc. of solutions containing 1 millimol in 10 cc., in tests II, III and IV, 0.05 cc. of solutions containing $^1/_8$ millimol in 10 cc., was mixed with 0.2 cc. of the test antigens (prepared with chicken serum) diluted 1:500, and 0.1 cc. of metanilic acid immune serum (No. 2). The control tube contains only antigen and immune serum.

Readings were taken after 20 minutes (first line) and 1 hour (second line) at room temperature, and after standing overnight in the ice box (third line).

Test antigens made from....	I. Metanilic acid					II. *o*-Aminobenzene-sulfonic acid			III. *m*-Aminophenylarsenic acid			IV. *m*-Aminobenzoic acid		
Substances used for inhibition tests.........	1	2	3	4	Control	1	2	Control	2	3	Control	2	4	Control
	f. tr.	0	++	+±	++	f. tr.	f. tr.	+±	0	±	+	0	±	+
	±	tr.	++	+±	++±	tr.	f. tr.	++	0	±	+	0	±	+
	++	+±	+++±	+++	+++±	+±	±	++±	±	+±	++	+	+±	++

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TABLE IX

Neutral solutions were prepared of the *p*-nitro derivatives of glutaranilic (1), adipanilic (2), pimelanilic (3), suberanilic (4) and sebacanilic acid (5), as described in Table VIII (C designates control). In tests II and VII, 0.05 cc. of the solutions containing $^1/_4$ millimol in 10 cc., in tests III, IV, V, VI and VIII, 0.05 cc. solutions containing $^1/_{16}$ millimol in 10 cc., and in test I, 0.05 cc. of solutions containing $^1/_{32}$ millimol in 10 cc. were mixed with 0.2 cc. of the test antigens (prepared with casein) diluted 1:500 and 2 drops of adipanilic acid or suberanilic acid immune serum.

Readings were taken after 15 minutes (first line) and 1½ hours (second line) at room temperature.

	Adipanilic acid immune serum and test antigens made from																			
	I. *p*-Aminoglutaranilic acid					II. *p*-Aminoadipanilic acid					III. *p*-Aminopimelanilic acid					IV. *p*-Aminosuberanilic acid				
Substances used for inhibition tests.........	1	2	3	4	C	1	2	3	4	C	1	2	3	4	C	1	2	3	4	C
	tr.	f. tr.	tr.	$\underline{\text{tr.}}$	$\underline{+}$	±	0	0	tr.	++	±	0	f. tr.	tr.	+	±	0	f. tr.	f. tr.	+
	tr.	f. tr.	$\underline{\text{tr.}}$	±	+	+	0	tr.	±	++±	+	f. tr.	tr.	$\underline{\text{tr.}}$	++	$\underline{+}$	f. tr.	tr.	tr.	+±

	Suberanilic acid immune serum (No. 1) and test antigens made from																			
	V. *p*-Aminoadipanilic acid					VI. *p*-Aminopimelanilic acid					VII. *p*-Aminosuberanilic acid					VIII. *p*-Aminosebacanilic acid				
Substances used for inhibition tests.........	2	3	4	5	C	2	3	4	5	C	2	3	4	5	C	2	3	4	5	C
	f. tr.	0	0	0	+	+	tr.	0	tr.	+±	+	±	0	f. tr.	++	+	tr.	0	0	+±
	±	tr.	0	f. tr.	+±	+	±	tr.	±	++	++	+	0	$\underline{\text{tr.}}$	++±	+±	$\underline{+}$	0	tr.	++

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TABLES X*a* AND X*b*

Neutral solutions were prepared of the *p*-nitrobenzoyl derivatives of glycine (G), *d,l*-leucine (L), glycyl-glycine (GG), glycyl-*d,l*-leucine (GL), *d,l*-leucyl-glycine (LG) and *d,l*-leucyl-*d,l*-leucine A (LL), as described in Table VIII (C designates control).

For the inhibition tests 0.05 cc. of the solutions (the concentration in millimols per 10 cc. is indicated in the tables) was mixed with 0.2 cc. of the test antigens (prepared from chicken serum) diluted 1:500 and two drops of immune serum.

TABLE X*a*

Immune sera for azoproteins made from *p*-aminobenzoyl peptides	Test antigens	G				GG							LG			
	Substances tested for inhibition	G	GG	LG	C	G	L	GG	GL	LG	LL	C	G	GG	LG	C
		Concentration 1:4				Concentration 1:4							Concentration 1:64			
G		0 0	+± +±	+± ++	++ ++±	0 0	+ +±	tr. tr.	+ +±	± ±	+ +±	+ +±	0 0	± ±	tr. ±	± +
		Concentration 1:16				Concentration 1:4							Concentration 1:4			
LG		tr. +	± +±	0 tr.	+± ++	+± ++	++ ++±	tr. +	++ ++±	0 tr.	++ ++±	++ ++±	++ ++±	++ ++±	0 tr.	++± +++

TABLE X*b*

Immune sera for azoproteins made from *p*-aminobenzoyl peptides	Test antigens	L					GG							GL					LL				
	Substances tested for inhibition	L	GG	GL	LL	C	G	L	GG	GL	LG	LL	C	L	GG	GL	LL	C	L	GG	GL	LL	C
		Concentration 1:4												Concentration 1:16					Concentration 1:32				
L		0 0	++ ++±	+ +±	± +±	++ ++±								0 0	++ ++±	± +±	± +±	++ ++±	0 0	± ++	0 tr.	0 f. tr.	± ++
		Concentration 1:16					Concentration 1:64							Concentration 1:4					Concentration 1:64				
GL		f. tr. ±	+± ++	0 0	0 tr.	+± ++	+ +±	+ +±	± ±	0 0	+ +±	+ +±	+ +±	++ ++±	++ +++	0 0	+± ++±	++± +++	+ +	+ +±	0 0	tr. +	+ +±

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As is seen from Tables VIII, IX, X *a* and X *b* the reactions on heterologous antigens are regularly inhibited more strongly, or not less, by the simple substances corresponding to the specific part of the homologous antigen than by heterologous substances, *e.g.* the reaction of metanilic acid immune sera on *m*-arsanilic acid antigen was more inhibited by metanilic acid than by *m*-arsanilic acid, etc.

In inhibition tests made with sera which previously had been absorbed with an heterologous antigen it was found that the reactions of such absorbed sera with the homologous antigen were still inhibited by the simple substances corresponding to the heterologous antigen used for absorption. This shows that the remaining antibodies have affinity for the absorbing antigen, although no precipitation occurs.

DISCUSSION

From the experiments presented one may conclude that the increase in specificity after absorption of azoprotein immune sera with heterologous antigens is referable to a multiplicity of antibodies. Particularly striking is the fact—which cannot well be understood on the assumption of a single antibody—that by exhaustion with various heterologous antigens fluids of different specificities can be prepared. The objection that some of the absorbing antigen may pass into solution and interfere with the reaction seems to be ruled out by the insolubility of the antigens made from stromata; of course, an alteration of antibodies by mere contact with the insoluble material would seem highly improbable.

As already mentioned, when results similar to those described are obtained with natural antigens, it is usually concluded that the antibodies present in one immune serum are severally directed toward distinct groupings or components of the antigen. This view while possible in cases of natural antigens is not tenable in those under discussion. For example, the cross reactions of metanilic acid immune sera are evidently due either to the presence of SO_3H or to acid groups in meta position (see Table III). But the antisera are not strictly specific for the SO_3H radical itself since they do not react with all aromatic sulfonic acids; likewise meta position of substituents, regardless of their nature, is not sufficient to cause a positive reaction. Thus it appears that the specificity is determined by the structure of the metanilic acid

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molecule as a whole. Clearly, there are not several specific groupings in the immunizing antigen, identical with groupings contained in the positively reacting heterologous antigens, to which the antibodies could be specifically related. The same consideration holds for the reactions of suberanilic acid immune sera on sebacanilic acid antigen.

The view just outlined is supported by the inhibition tests described. The reactions show that, although the immune sera are made up of fractions somewhat different in their specificity, *i.e.* in the affinity to various heterologous antigens, the antibodies all have maximal affinity for the immunizing antigens, the cross reactions, as a rule, being inhibited best by the homologous simple substances.

It can be concluded therefore, that the separation from an immune serum of antibody fractions, different in specificity, is no proof of the existence of several substances or special groups in the immunizing antigen. In other words, antibodies formed in response to one antigen, although adjusted to a certain structure, are not entirely uniform but vary in specificity to some degree. An additional argument in favor of this interpretation is the fact established repeatedly, that immune sera produced in animals of the same species may differ in their reactions with heterologous antigens. In this connection reference may be made to the presence in one serum of antibodies varying in their avidity as shown by the influence of temperature on the reactions.

The results under discussion have some bearing on absorption experiments with natural antigens. Thus one must expect that a separation of antibody fractions will be possible even when an inciting natural antigen does not contain several determinant structures; this might explain why such effects are to some extent obtained almost regularly. Evidence to show that the demonstration of divers antibody fractions is no decisive proof of the presence of several separable substances in the corresponding antigen has been brought forward by one of the authors and Furth (11), and by Burnet (12).

If the complexity of an antigen cannot be established chemically in individual cases it will not always be easy to determine the cause for the production of multiple antibodies, following the injection of an antigenic material. The assumption of several substances or determinant groups will be less probable if on continued exhaustion with

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an heterologous substance the homologous reaction tends to disappear, as was mostly the case in the experiments here reported.

SUMMARY

The phenomena observed on absorption of immune sera to azoproteins with heterologous azoantigens lead to the conclusion that in general the sera do not contain a single antibody but antibody fractions somewhat different in their reactivity for heterologous antigens. From the constitution of the azocomponent it follows that these fractions cannot be specific for distinct chemical groups in the molecule. In fact, inhibition experiments showed that in the cases examined the various antibodies are all specifically directed towards the whole molecule of the homologous hapten.

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